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(54) Title: MOLECULAR ANTIGEN ARRAY

(57) Abstract: The invention provides compositions and processes for the production of ordered and repetitive antigen or antigenic determinant arrays. The compositions of the invention are useful for the production of vaccines for the prevention of infectious diseases, the treatment of allergies and the treatment of cancers. Various embodiments of the invention provide for a core particle that is coated with any desired antigen in a highly ordered and repetitive fashion as the result of specific interactions.

## MOLECULAR ANTIGEN ARRAY

## BACKGROUND OF THE INVENTION

## Field of the Invention

[0001] The present invention is related to the fields of molecular biology, virology, immunology and medicine. The invention provides a composition comprising an ordered and repetitive antigen or antigenic determinant array. The invention also provides a process for producing an antigen or antigenic determinant in an ordered and repetitive array. The ordered and repetitive antigen or antigenic determinant is useful in the production of vaccines for the treatment of infectious diseases, the treatment of allergies and as a pharmaccine to prevent or cure cancer and to generate defined self-specific antibodies and specific immune responses of the Th2 type.

## Background Art

[0002] Vaccine development for the prevention of infectious disease has had the greatest impact on human health of any medical invention. It is estimated that three million deaths per year are prevented worldwide by vaccination (Hillemann, *Nature Medicine* 4:507 (1998)). The most common vaccination strategy, the use of attenuated (*i.e.*, less virulent) pathogens or closely related organisms, was first demonstrated by Edward Jenner in 1796, who vaccinated against smallpox by the administration of a less dangerous cowpox virus. Although a number of live attenuated viruses (*e.g.*, measles, mumps, rubella, varicella, adenovirus, polio, influenza) and bacteria (*e.g.*, bacille Calmette-Guerin (BCG) against tuberculosis) are successfully administered for vaccination, there is a risk for the development of serious complications related to a reversion to virulence and infection by the 'vaccine' organism, in particular in immunocompromised individuals.

[0003] The specific design of attenuated viruses is now enabled by recombinant DNA technology (*i.e.*, genetic engineering) through the generation of deletion or

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mutation variants. For example, the administration of an engineered Simian Immunodeficiency Virus (SIV) with a deletion within the *nef* gene was shown to protect macaques from subsequent infection with a pathogenic SIV strain (Daniel *et al.*, *Science* 258:1938-1941 (1992)). However, the progression of acquired immunodeficiency syndrome (AIDS)-like symptoms in animals administered attenuated SIV raises safety concerns (Baba *et al.*, *Science* 267:1820-1825 (1995)).

[0004] As an alternative approach, attenuated viruses or bacteria may be used as carriers for the antigen-encoding genes of a pathogen that is considered too unsafe to be administered in an attenuated form (*e.g.*, Human Immunodeficiency Virus (HIV)). Upon delivery of the antigen-encoding gene to the host, the antigen is synthesized *in situ*. Vaccinia and related avipox viruses have been used as such carriers for various genes in preclinical and clinical studies for a variety of diseases (*e.g.*, Shen *et al.*, *Science* 252:440 (1991)). One disadvantage of this vaccination strategy is that it does not mimic the virion surface, because the recombinant protein is expressed on the surface of the host cell. Additionally, complications may develop in immunocompromised individuals, as evidenced by life-threatening disseminated vaccinia infections (Redfield, *N. Eng. J. Med.* 316:673 (1998)).

[0005] A fourth vaccination approach involves the use of isolated components of a pathogen, either purified from the pathogen grown *in vitro* (*e.g.*, influenza hemagglutinin or neuraminidase) or after heterologous expression of a single viral protein (*e.g.*, Hepatitis B surface antigen). For example, recombinant, mutated toxins (detoxified) are used for vaccination against diphtheria, tetanus, cholera and pertussis toxins (Levine *et al.*, *New generation vaccines*, 2nd edn., Marcel Dekker, Inc., New York 1997), and recombinant proteins of HIV (gp120 and full-length gp160) were evaluated as a means to induce neutralizing antibodies against HIV with disappointing results (Connor *et al.*, *J. Virol.* 72:1552 (1998)). Recently, promising results were obtained with soluble oligomeric gp160, that can induce CTL response and elicit antibodies with neutralizing activity against HIV-1 isolates (Van Cortt *et al.*, *J. Virol.* 71:4319 (1997)). In addition, peptide vaccines

may be used in which known B- or T-cell epitopes of an antigen are coupled to a carrier molecule designed to increase the immunogenicity of the epitope by stimulating T-cell help. However, one significant problem with this approach is that it provides a limited immune response to the protein as a whole. Moreover, vaccines have to be individually designed for different MHC haplotypes. The most serious concern for this type of vaccine is that protective antiviral antibodies recognize complex, three-dimensional structures that cannot be mimicked by peptides.

[0006] A more novel vaccination strategy is the use of DNA vaccines (Donnelly *et al.*, *Ann. Rev. Immunol.* 15:617 (1997)), which may generate MHC Class I-restricted CTL responses (without the use of a live vector). This may provide broader protection against different strains of a virus by targeting epitopes from conserved internal proteins pertinent to many strains of the same virus. Since the antigen is produced with mammalian post-translational modification, conformation and oligomerization, it is more likely to be similar or identical to the wild-type protein produced by viral infection than recombinant or chemically modified proteins. However, this distinction may turn out to be a disadvantage for the application of bacterial antigens, since non-native post-translational modification may result in reduced immunogenicity. In addition, viral surface proteins are not highly organized in the absence of matrix proteins.

[0007] In addition to applications for the prevention of infectious disease, vaccine technology is now being utilized to address immune problems associated with allergies. In allergic individuals, antibodies of the IgE isotype are produced in an inappropriate humoral immune response towards particular antigens (allergens). The treatment of allergies by allergy immunotherapy requires weekly administration of successively increasing doses of the particular allergen over a period of up to 3-5 years. Presumably, 'blocking' IgG antibodies are generated that intercept allergens in nasal or respiratory secretions or in membranes before they react with IgE antibodies on mast cells. However, no constant relationship exists between IgG titers and symptom relief. Presently, this is an extremely time-



and cost-consuming process, to be considered only for patients with severe symptoms over an extended period each year.

[0008] It is well established that the administration of purified proteins alone is usually not sufficient to elicit a strong immune response; isolated antigen generally must be given together with helper substances called adjuvants. Within these adjuvants, the administered antigen is protected against rapid degradation, and the adjuvant provides an extended release of a low level of antigen.

[0009] Unlike isolated proteins, viruses induce prompt and efficient immune responses in the absence of any adjuvants both with and without T-cell help (Bachmann & Zinkernagel, *Ann. Rev. Immunol.* 15:235-270 (1997)). Although viruses often consist of few proteins, they are able to trigger much stronger immune responses than their isolated components. For B cell responses, it is known that one crucial factor for the immunogenicity of viruses is the repetitiveness and order of surface epitopes. Many viruses exhibit a quasi-crystalline surface that displays a regular array of epitopes which efficiently crosslinks epitope-specific immunoglobulins on B cells (Bachmann & Zinkernagel, *Immunol. Today* 17:553-558 (1996)). This crosslinking of surface immunoglobulins on B cells is a strong activation signal that directly induces cell-cycle progression and the production of IgM antibodies. Further, such triggered B cells are able to activate T helper cells, which in turn induce a switch from IgM to IgG antibody production in B cells and the generation of long-lived B cell memory - the goal of any vaccination (Bachmann & Zinkernagel, *Ann. Rev. Immunol.* 15:235-270 (1997)). Viral structure is even linked to the generation of anti-antibodies in autoimmune disease and as a part of the natural response to pathogens (see Fehr, T., *et al.*, *J. Exp. Med.* 185:1785-1792 (1997)). Thus, antigens on viral particles that are organized in an ordered and repetitive array are highly immunogenic since they can directly activate B cells.

[0010] In addition to strong B cell responses, viral particles are also able to induce the generation of a cytotoxic T cell response, another crucial arm of the immune system. These cytotoxic T cells are particularly important for the elimination of

non-cytopathic viruses such as HIV or Hepatitis B virus and for the eradication of tumors. Cytotoxic T cells do not recognize native antigens but rather recognize their degradation products in association with MHC class I molecules (Townsend & Bodmer, *Ann. Rev. Immunol.* 7:601-624 (1989)). Macrophages and dendritic cells are able to take up and process exogenous viral particles (but not their soluble, isolated components) and present the generated degradation product to cytotoxic T cells, leading to their activation and proliferation (Kovacsovics-Bankowski *et al.*, *Proc. Natl. Acad. Sci. USA* 90:4942-4946 (1993); Bachmann *et al.*, *Eur. J. Immunol.* 26:2595-2600 (1996)).

[0011] Viral particles as antigens exhibit two advantages over their isolated components: (1) Due to their highly repetitive surface structure, they are able to directly activate B cells, leading to high antibody titers and long-lasting B cell memory; and (2) Viral particles but not soluble proteins are able to induce a cytotoxic T cell response, even if the viruses are non-infectious and adjuvants are absent.

[0012] Several new vaccine strategies exploit the inherent immunogenicity of viruses. Some of these approaches focus on the particulate nature of the virus particle; for example *see* Harding, C.V. and Song, R., (*J. Immunology* 153:4925 (1994)), which discloses a vaccine consisting of latex beads and antigen; Kovacsovics-Bankowski, M., *et al.* (*Proc. Natl. Acad. Sci. USA* 90:4942-4946 (1993)), which discloses a vaccine consisting of iron oxide beads and antigen; U.S. Patent No 5,334,394 to Kossovsky, N., *et al.*, which discloses core particles coated with antigen; U.S. Patent No. 5,871,747, which discloses synthetic polymer particles carrying on the surface one or more proteins covalently bonded thereto; and a core particle with a non-covalently bound coating, which at least partially covers the surface of said core particle, and at least one biologically active agent in contact with said coated core particle (*see, e.g.*, WO 94/15585).

[0013] However, a disadvantage of these viral mimicry systems is that they are not able to recreate the ordered presentation of antigen found on the viral surface. Antigens coupled to a surface in a random orientation are found to induce CTL

response and no or only weak B-cell response. For an efficient vaccine, both arms of the immune system have to be strongly activated, as described above and in Bachmann & Zinkernagel, *Ann. Rev. Immunol.* 15:235 (1997).

[0014] In another example, recombinant viruses are being utilized for antigen delivery. Filamentous phage virus containing an antigen fused to a capsid protein has been found to be highly immunogenic (*see* Perham R.N., *et al.*, *FEMS Microbiol. Rev.* 17:25-31 (1995); Willis *et al.*, *Gene* 128:85-88 (1993); Minenkova *et al.*, *Gene* 128:85-88 (1993)). However, this system is limited to very small peptides (5 or 6 amino acid residues) when the fusion protein is expressed at a high level (Iannolo *et al.*, *J. Mol. Biol.* 248:835-844 (1995)) or limited to the low level expression of larger proteins (de la Cruz *et al.*, *J. Biol. Chem.* 263:4318-4322 (1988)). For small peptides, so far only the CTL response is observed and no or only weak B-cell response.

[0015] In yet another system, recombinant alphaviruses are proposed as a means of antigen delivery (*see* U.S. Patent Nos. 5,766,602; 5,792,462; 5,739,026; 5,789,245 and 5,814,482). Problems with the recombinant virus systems described so far include a low density expression of the heterologous protein on the viral surface and/or the difficulty of successfully and repeatedly creating a new and different recombinant viruses for different applications.

[0016] In a further development, virus-like particles (VLPs) are being exploited in the area of vaccine production because of both their structural properties and their non-infectious nature. VLPs are supermolecular structures built in a symmetric manner from many protein molecules of one or more types. They lack the viral genome and, therefore, are noninfectious. VLPs can often be produced in large quantities by heterologous expression and can be easily be purified.

[0017] Examples of VLPs include the capsid proteins of Hepatitis B virus (Ulrich, *et al.*, *Virus Res.* 50:141-182 (1998)), measles virus (Warnes, *et al.*, *Gene* 160:173-178 (1995)), Sindbis virus, rotavirus (U.S. Patent Nos. 5,071,651 and 5,374,426), foot-and-mouth-disease virus (Twomey, *et al.*, *Vaccine* 13:1603-1610, (1995)), Norwalk virus (Jiang, X., *et al.*, *Science* 250:1580-1583

(1990); Matsui, S.M., *et al.*, *J. Clin. Invest.* 87:1456-1461 (1991)), the retroviral GAG protein (PCT Patent Appl. No. WO 96/30523), the retrotransposon Ty protein p1, the surface protein of Hepatitis B virus (WO 92/11291) and human papilloma virus (WO 98/15631). In some instances, recombinant DNA technology may be utilized to fuse a heterologous protein to a VLP protein (Kratz, P.A., *et al.*, *Proc. Natl. Acad. Sci. USA* 96: 19151920 (1999)).

[0018] Thus, there is a need in the art for the development of new and improved vaccines that promote a strong CTL and B-cell immune response as efficiently as natural pathogens.

#### BRIEF SUMMARY OF THE INVENTION

[0019] The invention provides a versatile new technology that allows production of particles or pili coated with any desired antigen. The technology allows the creation of highly efficient vaccines against infectious diseases and for the creation of vaccines for the treatment of allergies and cancers. The invention also provides compositions suited for the induction of Th type 2 T-helper cells (Th2 cells). Thus, efficient vaccines for the treatment of chronic diseases induced or accelerated by a Th1 type immune response, such as arthritis, colitis, diabetes and multiple sclerosis can be produced with the technology provided by this invention.

[0020] In a first embodiment, the invention provides a novel composition comprising (A) a non-natural molecular scaffold and (B) an antigen or antigenic determinant.

[0021] The non-natural molecular scaffold comprises, or alternatively consists of, (i) a core particle selected from the group consisting of (1) a core particle of non-natural origin and (2) a core particle of natural origin; and (ii) an organizer comprising at least one first attachment site, wherein said organizer is connected to said core particle by at least one covalent bond.

[0022] In certain specific embodiments, the core particle naturally contains an organizer. One example of an embodiment of the invention where the organizer

is naturally occurring is the bacterial pilus or pilin protein. The antigenic determinant may be linked by a cysteine to a naturally occurring lysine residue of the bacterial pili or pilin protein.

[0023] The antigen or antigenic determinant has at least one second attachment site which is selected from the group consisting of (i) an attachment site not naturally occurring with said antigen or antigenic determinant; and (ii) an attachment site naturally occurring with said antigen or antigenic determinant.

[0024] The invention provides for an ordered and repetitive antigen array through an association of the second attachment site to the first attachment site by way of at least one non-peptide bond. Thus, the antigen or antigenic determinant and the non-natural molecular scaffold are brought together through this association of the first and the second attachment site to form an ordered and repetitive antigen array.

[0025] In another embodiment, the core particle of the aforementioned composition comprises a virus, a virus-like particle, a bacterial pilus, a structure formed from bacterial pilin, a bacteriophage, a viral capsid particle or a recombinant form thereof. Alternatively, the core particle may be a synthetic polymer or a metal.

[0026] In yet another embodiment, the core particle comprises, or alternatively consists of, one or more different Hepatitis core (capsid) proteins (HBcAgs). In a related embodiment, one or more cysteine residues of these HBcAgs are either deleted or substituted with another amino acid residue (*e.g.*, a serine residue). In a specific embodiment, the cysteine residues of the HBcAg used to prepare compositions of the invention which correspond to amino acid residues 48 and 107 in SEQ ID NO:134 are either deleted or substituted with another amino acid residue (*e.g.*, a serine residue).

[0027] Further, the HBcAg variants used to prepare compositions of the invention will generally be variants which retain the ability to associate with other HBcAgs to form dimeric or multimeric structures that present ordered and repetitive antigen or antigenic determinant arrays.

[0028] In another embodiment, the non-natural molecular scaffold comprises, or alternatively consists of, pili or pilus-like structures that have been either produced from pilin proteins or harvested from bacteria. When pili or pilus-like structures are used to prepare compositions of the invention, they may be formed from products of pilin genes which are naturally resident in the bacterial cells but have been modified by genetically engineered (*e.g.*, by homologous recombination) or pilin genes which have been introduced into these cells.

[0029] In a related embodiment, the core particle comprises, or alternatively consists of, pili or pilus-like structures that have been either prepared from pilin proteins or harvested from bacteria. These core particles may be formed from products of pilin genes naturally resident in the bacterial cells. Further, antigens or antigenic determinants may be linked to these core particles naturally containing an organizer. In such a case, the core particles will generally be linked to a second attachment site of the antigen or antigenic determinant. In most embodiments of the invention, the pili or pilus-like structures will be able to form an ordered and repetitive antigen array with the antigen or antigenic determinant linked to the core particle at a specific or preferred location (*e.g.*, a specific amino acid residue).

[0030] In a particular embodiment, the organizer may comprise at least one first attachment site. The first and the second attachment sites are particularly important elements of compositions of the invention. In various embodiments of the invention, the first and/or the second attachment site may be an antigen and an antibody or antibody fragment thereto; biotin and avidin; streptavidin and biotin; a receptor and its ligand; a ligand-binding protein and its ligand; interacting leucine zipper polypeptides; an amino group and a chemical group reactive thereto; a carboxyl group and a chemical group reactive thereto; a sulfhydryl group and a chemical group reactive thereto; or a combination thereof.

[0031] In one embodiment, the invention provides the coupling of almost any antigen of choice to the surface of a virus, bacterial pilus, structure formed from bacterial pilin, bacteriophage, virus-like particle or viral capsid particle. By

bringing an antigen into a quasi-crystalline 'virus-like' structure, the invention exploits the strong antiviral immune reaction of a host for the production of a highly efficient immune response, *i.e.*, a vaccination, against the displayed antigen.

[0032] In another embodiment, the core particle may be selected from the group consisting of: recombinant proteins of Rotavirus, recombinant proteins of Norwalk virus, recombinant proteins of Alphavirus, recombinant proteins of Foot and Mouth Disease virus, recombinant proteins of Retrovirus, recombinant proteins of Hepatitis B virus, recombinant proteins of Tobacco mosaic virus, recombinant proteins of Flock House Virus, and recombinant proteins of human Papillomavirus.

[0033] In yet another embodiment, the antigen may be selected from the group consisting of: (1) a protein suited to induce an immune response against cancer cells; (2) a protein suited to induce an immune response against infectious diseases; (3) a protein suited to induce an immune response against allergens; and (4) a protein suited to induce an immune response in pets or farm animals.

[0034] In one embodiment, the invention relates to the induction of specific Th type 2 T-helper cells (Th2 cells) using antigens attached to Pili. The induction of Th2 responses may be beneficial for the treatment of a number of diseases. For example, many chronic diseases in humans and animals, such as arthritis, colitis, diabetes and multiple sclerosis are dominated by Th1 response, where T cells secrete IFN $\gamma$  and other pro-inflammatory cytokines precipitating disease.

[0035] In a particular embodiment of the invention, the first attachment site and/or the second attachment site comprise an interacting leucine zipper polypeptide. In a related embodiment, the first attachment site and/or the second attachment site are selected from the group comprising: (1) the *JUN* leucine zipper protein domain; and (2) the *FOS* leucine zipper protein domain.

[0036] In another embodiment, the first attachment site and/or the second attachment site are selected from the group comprising: (1) a genetically engineered lysine residue and (2) a genetically engineered cysteine residue, two residues that may be chemically linked together.

[0037] The invention also includes embodiments where the organizer particle has only a single first attachment site and the antigen or antigenic determinant has only a single second attachment site. Thus, when an ordered and repetitive antigen array is prepared using such embodiments, each organizer will be bound to a single antigen or antigenic determinant.

[0038] In one aspect, the invention provides compositions comprising, or alternatively consisting of, (a) a non-natural molecular scaffold comprising (i) a core particle selected from the group consisting of a core particle of non-natural origin and a core particle of natural origin, and (ii) an organizer comprising at least one first attachment site, wherein the core particle comprises, or alternatively consists of, a bacterial pilus, a pilus-like structure, or a modified HBcAg, or fragment thereof, and wherein the organizer is connected to the core particle by at least one covalent bond, and (b) an antigen or antigenic determinant with at least one second attachment site, the second attachment site being selected from the group consisting of (i) an attachment site not naturally occurring with the antigen or antigenic determinant and (ii) an attachment site naturally occurring with the antigen or antigenic determinant, wherein the second attachment site is capable of association through at least one non-peptide bond to the first attachment site, and wherein the antigen or antigenic determinant and the scaffold interact through the association to form an ordered and repetitive antigen array.

[0039] Other embodiments of the invention include processes for the production of compositions of the invention and a methods of medical treatment using vaccine compositions described herein.

[0040] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are intended to provide further explanation of the invention as claimed.



## BRIEF DESCRIPTION OF THE DRAWINGS

- [0041] Figure 1 shows a Western blot demonstrating the production of viral particles containing the E2-*JUN* fusion protein using the pCYTs::E2*JUN* expression vector.
- [0042] Figure 2 shows a Western blot demonstrating the production of viral particles containing the E2-*JUN* fusion protein expressed from pTE5'2J::E2*JUN* expression vector.
- [0043] Figure 3 shows a Western dot blot demonstrating bacterial and eukaryotic expression of the *FOS*-hgh antigen.
- [0044] Figure 4 shows the expression of HBcAg-JUN in *E. coli* cells.
- [0045] Figure 5 shows a Western blot demonstrating that HBcAg-JUN is soluble in *E. coli* lysates.
- [0046] Figure 6 shows an SDS-PAGE analysis of enrichment of HBcAg-JUN capsid particles on a sucrose density gradient.
- [0047] Figure 7 shows a non-reducing SDS-PAGE analysis of the coupling of hGH-FOS and HBcAg-JUN particles.
- [0048] Figure 8 depicts an analysis by SDS-PAGE of the coupling reaction of the FLAG peptide to HBcAG-Lys treated with iodacetamide and activated with Sulfo-MBS. The excess of cross-linker and of peptide over HBcAg-Lys monomer is indicated below the figure.
- [0049] Figure 9 depicts an analysis of coupling of the FLAG peptide to type-1 bacterial pili by SDS-PAGE. Lane 1 shows the unreacted pili subunit FimA. Lane 3 shows the purified reaction mixture of the pili with the FLAG peptide. The upper band corresponds to the coupled product, while the lower band corresponds to the unreacted subunit.
- [0050] Figure 10 depicts an analysis by SDS-PAGE of the derivatization of HBcAg-Lys with SPDP.
- [0051] Figure 11 depicts an analysis by SDS-PAGE of the derivatization of HBcAg-Lys with Sulfo-MBS.

- [0052] Figure 12 depicts an analysis by SDS-PAGE of the coupling of HBcAg-Lys-2cyc-Mut to the FLAG peptide. The arrow shows the bands corresponding to the coupling of one and two FLAG peptides, respectively, to one subunit of HBcAg-Lys-2cyc-Mut. Lane M corresponds to the marker, lane 1 to the unreacted HBcAg-Lys-2cyc-Mut, lane 2 to HBcAg-Lys-2cyc-Mut activated with Sulfo-MBS, and lane 3 activated HBcAg-Lys-2cyc-Mut after reaction with the FLAG peptide containing an N-terminal cysteine.
- [0053] Figure 13 depicts an analysis by SDS-PAGE of the coupling of pili to the p33 peptide.
- [0054] Figure 14A shows an analysis of coupling of DP178c peptide by SDS-PAGE analysis and Coomassie staining. Lane 1 corresponds to the supernatant of the coupling reaction after centrifugation, while lane 2 corresponds to the pellet. Figure 14B shows an ELISA data and subtype analysis of mice sera immunized with Pili-DP178c. The OD (450 nm) of the ELISA signal obtained at a fifty-fold dilution of the sera is shown in the diagram. For each subtype determination, mice sera were titrated from a fifty-fold dilution in two-fold dilution steps. The ELISA titer of the IgG1 subtype (OD50 dilution) was 1:400, while the titer of the IgG2b subtype was 1:100. The other subtypes all had titers inferior to 1:50. The IgG isotype pattern is characteristic of a Th2 response, with a high IgG1 titer and a low IgG2a titer.
- [0055] Figure 15A shows an analysis of Coupling of GRA2 to Pili by SDS-PAGE analysis and Coomassie staining. Figure 15B relates to immunization of mice with Pili-GRA2 and IgG subtype determination. Depicted is an analysis of total IgG titer and IgG subtype titers by ELISA. The ELISA titer is given by the dilution of sera at which OD50 is obtained. The result of the immunization of two individual mice is shown. A high IgG1 titer and a low IgG2a titer is characteristic of a Th2 response.
- [0056] Figure 16A shows an analysis of coupling of B2 and D2 peptides to Pili by SDS-PAGE analysis and Coomassie staining. Figure 16B relates to immunization of mice with Pili-B2 and IgG subtype determination. The OD (450

nm) of the ELISA signal obtained at a fifty-fold dilution of the sera is shown in the diagram. For each subtype determination, mice sera were titrated from a fifty-fold dilution in two-fold dilution steps. The titer of the IgG1 subtype (dilution at which the signal corresponds to OD 50) was 1:250, while the other subtypes all had titers inferior to 1:50. The titer of the IgG1 subtype is much higher than the titer of the IgG2a subtype, a pattern typical for a Th2 response.

[0057] Figure 17 relates to the measurement of antibodies specific for TNF $\alpha$  protein in the serum of mice immunized with the muTNF $\alpha$  peptide coupled to type-1 Pili. As a control, preimmune sera of two mice were assayed for binding to TNF $\alpha$  protein. Sera were added at three different dilutions (1:50, 1:100 and 1:200), and bound IgG was detected with a horseradish peroxidase-conjugated anti-murine IgG antibody. Results from four individual mice are shown on day 21 and day 43. OD (450 nm): optical density at 450 nm.

[0058] Figure 18A shows an analysis of coupling of 5'-TNF II and 3'-TNF II by SDS-PAGE and Coomassie staining. Lane M is the marker lane. Untreated Pili were loaded on lane 1, Pili-5'-TNF II before dialysis on lane 2, Pili-3'-TNF II before dialysis on lane 3, Pili-5'-TNF II after dialysis on lane 4, pili-3'-TNF II after dialysis on lane 5. The arrow indicates the size at which the coupled product migrates.

[0059] Figure 18B shows an ELISA analysis of sera of mice immunized with Pili-5'-TNF II and Pili-3'-TNF II: Anti-TNF $\alpha$  ELISA. IgG antibodies specific for native TNF $\alpha$  protein were measured in a specific ELISA. 2  $\mu$ g/ml native TNF $\alpha$  protein was coated on ELISA plates. Sera were added at different dilutions and bound IgG was detected with a horseradish peroxidase-conjugated anti-murine IgG antibody. Results from four individual mice are shown on day 21 and day 43 OD (450 nm): optical density at 450 nm. The data show that mice immunized with the TNF peptides coupled to pili mount an antibody response against native TNF $\alpha$  protein, thus breaking self-tolerance.

[0060] Figure 18C shows an ELISA analysis of sera of mice immunized with Pili-5'-TNF II and Pili-3'-TNF II: Anti-TNF $\alpha$  peptide ELISA. IgG antibodies specific

for the 5'TNF II and 3'TNF II peptides were measured in a specific ELISA: 10  $\mu$ g/ml Ribonuclease A coupled to 5'TNF II or 3'TNF II peptide was coated on ELISA plates. Sera were added at different dilutions and bound IgG was detected with a horseradish peroxidaseconjugated anti-murine IgG antibody. Results from four individual mice are shown on day 21.

[0061] Figure 18D shows that IgG subtype analysis of anti-TNF peptide antibodies in mice vaccinated with the corresponding TNF-peptides coupled to Pili. Results from four individual mice (no. 1-4) are shown on day 50. ELISA titer: dilution step at which half-maximal optical density was reached (-log 2 of 40-fold prediluted sera). The high IgG1 titer obtained as compared to the very low IgG2a titer is typical of a Th2 response.

[0062] Figure 19A shows an analysis of coupling of M2 peptide to Pili by SDS-PAGE analysis and Coomassie staining. The bands corresponding to non-coupled Pili and to the coupling product, Pili-M2, are indicated by arrows. Figure 19B shows an ELISA analysis and IgG subtype determination of mice vaccinated with Pili-M2. Sera were diluted eighty-fold, and titrated down in two-fold dilution steps. For the IgG1 subtype, a titer of 1:2560 was obtained, while for the IgG2a and IgG2b subtypes, titers below 1:100 were obtained. The titer for the IgG3 subtype was below 1:80. Titers were calculated as the serum dilution resulting in half-maximal optical density ( $OD_{50}$ ). A strong IgG1 titer in conjunction with a low IgG2a titer is characteristic for a Th2 type response. Average results from two mice are shown as optical densities obtained with a 1:80 dilution of the serum.

[0063] Figure 20 shows an ELISA analysis and IgG subtype determination of sera from mice immunized with HBcAg-Lys-2cys-Mut coupled to the Flag peptide. Ribonuclease A coupled to Flag peptide was coated at 10  $\mu$ g/ml, and serum was added at a 1:40 dilution. In contrast to experiments where mice were immunized with antigens coupled to Pili, there is no predominance of the IgG1 subtype over the other IgG subtypes.

## DETAILED DESCRIPTION OF THE INVENTION

## 1. Definitions

[0064] The following definitions are provided to clarify the subject matter which the inventors consider to be the present invention.

[0065] Alphavirus: As used herein, the term "alphavirus" refers to any of the RNA viruses included within the genus *Alphavirus*. Descriptions of the members of this genus are contained in Strauss and Strauss, *Microbiol. Rev.*, 58:491-562 (1994). Examples of alphaviruses include Aura virus, Bebaru virus, Cabassou virus, Chikungunya virus, Easter equine encephalomyelitis virus, Fort morgan virus, Getah virus, Kyzylagach virus, Mayoaro virus, Middleburg virus, Mucambo virus, Ndumu virus, Pixuna virus, Tonate virus, Trinita virus, Una virus, Western equine encephalomyelitis virus, Whataroa virus, Sindbis virus (SIN), Semliki forest virus (SFV), Venezuelan equine encephalomyelitis virus (VEE), and Ross River virus.

[0066] Antigen: As used herein, the term "antigen" is a molecule capable of being bound by an antibody. An antigen is additionally capable of inducing a humoral immune response and/or cellular immune response leading to the production of B- and/or T-lymphocytes. An antigen may have one or more epitopes (B- and T-epitopes). The specific reaction referred to above is meant to indicate that the antigen will react, in a highly selective manner, with its corresponding antibody and not with the multitude of other antibodies which may be evoked by other antigens.

[0067] Antigenic determinant: As used herein, the term "antigenic determinant" is meant to refer to that portion of an antigen that is specifically recognized by either B- or T-lymphocytes. B-lymphocytes respond to foreign antigenic determinants via antibody production, whereas T-lymphocytes are the mediator of cellular immunity. Thus, antigenic determinants or epitopes are those parts of an antigen that are recognized by antibodies, or in the context of an MHC, by T-cell receptors.

- [0068] Association: As used herein, the term "association" as it applies to the first and second attachment sites, is used to refer to at least one non-peptide bond. The nature of the association may be covalent, ionic, hydrophobic, polar or any combination thereof.
- [0069] Attachment Site, First: As used herein, the phrase "first attachment site" refers to an element of the "organizer", itself bound to the core particle in a non-random fashion, to which the second attachment site located on the antigen or antigenic determinant may associate. The first attachment site may be a protein, a polypeptide, an amino acid, a peptide, a sugar, a polynucleotide, a natural or synthetic polymer, a secondary metabolite or compound (biotin, fluorescein, retinol, digoxigenin, metal ions, phenylmethylsulfonylfluoride), or a combination thereof, or a chemically reactive group thereof. Multiple first attachment sites are present on the surface of the non-natural molecular scaffold in a repetitive configuration.
- [0070] Attachment Site, Second: As used herein, the phrase "second attachment site" refers to an element associated with the antigen or antigenic determinant to which the first attachment site of the "organizer" located on the surface of the non-natural molecular scaffold may associate. The second attachment site of the antigen or antigenic determinant may be a protein, a polypeptide, a peptide, a sugar, a polynucleotide, a natural or synthetic polymer, a secondary metabolite or compound (biotin, fluorescein, retinol, digoxigenin, metal ions, phenylmethylsulfonylfluoride), or a combination thereof, or a chemically reactive group thereof. At least one second attachment site is present on the antigen or antigenic determinant.
- [0071] Core particle: As used herein, the term "core particle" refers to a rigid structure with an inherent repetitive organization that provides a foundation for attachment of an "organizer". A core particle as used herein may be the product of a synthetic process or the product of a biological process.
- [0072] In certain embodiments of the invention, the antigens or antigenic determinants are directly linked to the core particle.

- [0073]       Cis-acting: As used herein, the phrase "*cis-acting*" sequence refers to nucleic acid sequences to which a replicase binds to catalyze the RNA-dependent replication of RNA molecules. These replication events result in the replication of the full-length and partial RNA molecules and, thus, the alphavirus subgenomic promoter is also a "*cis-acting*" sequence. *Cis-acting* sequences may be located at or near the 5' end, 3' end, or both ends of a nucleic acid molecule, as well as internally.
- [0074]       Fusion: As used herein, the term "fusion" refers to the combination of amino acid sequences of different origin in one polypeptide chain by in-frame combination of their coding nucleotide sequences. The term "fusion" explicitly encompasses internal fusions, *i.e.*, insertion of sequences of different origin within a polypeptide chain, in addition to fusion to one of its termini.
- [0075]       Heterologous sequence: As used herein, the term "heterologous sequence" refers to a second nucleotide sequence present in a vector of the invention. The term "heterologous sequence" also refers to any amino acid or RNA sequence encoded by a heterologous DNA sequence contained in a vector of the invention. Heterologous nucleotide sequences can encode proteins or RNA molecules normally expressed in the cell type in which they are present or molecules not normally expressed therein (*e.g.*, Sindbis structural proteins).
- [0076]       Isolated: As used herein, when the term "isolated" is used in reference to a molecule, the term means that the molecule has been removed from its native environment. For example, a polynucleotide or a polypeptide naturally present in a living animal is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated." Further, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Isolated RNA molecules include *in vivo* or *in vitro* RNA replication products of DNA and RNA molecules. Isolated nucleic acid molecules further include synthetically produced molecules. Additionally, vector molecules contained in recombinant host cells are also isolated. Thus, not all "isolated" molecules need be "purified."

- [0077] Immunotherapeutic: As used herein, the term "immunotherapeutic" is a composition for the treatment of diseases or disorders. More specifically, the term is used to refer to a method of treatment for allergies or a method of treatment for cancer.
- [0078] Individual: As used herein, the term "individual" refers to multicellular organisms and includes both plants and animals. Preferred multicellular organisms are animals, more preferred are vertebrates, even more preferred are mammals, and most preferred are humans.
- [0079] Low or undetectable: As used herein, the phrase "low or undetectable," when used in reference to gene expression level, refers to a level of expression which is either significantly lower than that seen when the gene is maximally induced (*e.g.*, at least five fold lower) or is not readily detectable by the methods used in the following examples section.
- [0080] Lectin: As used herein, proteins obtained particularly from the seeds of leguminous plants, but also from many other plant and animal sources, that have binding sites for specific mono- or oligosaccharides. Examples include concanavalin A and wheat-germ agglutinin, which are widely used as analytical and preparative agents in the study of glycoprotein.
- [0081] Natural origin: As used herein, the term "natural origin" means that the whole or parts thereof are not synthetic and exist or are produced in nature.
- [0082] Non-natural: As used herein, the term generally means not from nature, more specifically, the term means from the hand of man.
- [0083] Non-natural origin: As used herein, the term "non-natural origin" generally means synthetic or not from nature; more specifically, the term means from the hand of man.
- [0084] Non-natural molecular scaffold: As used herein, the phrase "non-natural molecular scaffold" refers to any product made by the hand of man that may serve to provide a rigid and repetitive array of first attachment sites. Ideally but not necessarily, these first attachment sites are in a geometric order. The non-natural molecular scaffold may be organic or non-organic and may be synthesized



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chemically or through a biological process, in part or in whole. The non-natural molecular scaffold is comprised of: (a) a core particle, either of natural or non-natural origin; and (b) an organizer, which itself comprises at least one first attachment site and is connected to a core particle by at least one covalent bond. In a particular embodiment, the non-natural molecular scaffold may be a virus, virus-like particle, a bacterial pilus, a virus capsid particle, a phage, a recombinant form thereof, or synthetic particle.

[0085] Ordered and repetitive antigen or antigenic determinant array: As used herein, the term "ordered and repetitive antigen or antigenic determinant array" generally refers to a repeating pattern of antigen or antigenic determinant, characterized by a uniform spacial arrangement of the antigens or antigenic determinants with respect to the non-natural molecular scaffold. In one embodiment of the invention, the repeating pattern may be a geometric pattern. Examples of suitable ordered and repetitive antigen or antigenic determinant arrays are those which possess strictly repetitive paracrystalline orders of antigens or antigenic determinants with spacings of 5 to 15 nanometers.

[0086] Organizer: As used herein, the term "organizer" is used to refer to an element bound to a core particle in a non-random fashion that provides a nucleation site for creating an ordered and repetitive antigen array. An organizer is any element comprising at least one first attachment site that is bound to a core particle by at least one covalent bond. An organizer may be a protein, a polypeptide, a peptide, an amino acid (*i.e.*, a residue of a protein, a polypeptide or peptide), a sugar, a polynucleotide, a natural or synthetic polymer, a secondary metabolite or compound (biotin, fluorescein, retinol, digoxigenin, metal ions, phenylmethylsulfonylfluoride), or a combination thereof, or a chemically reactive group thereof.

[0087] Permissive temperature: As used herein, the phrase "permissive temperature" refers to temperatures at which an enzyme has relatively high levels of catalytic activity.

- [0088] Pili: As used herein, the term "pili" (singular being "pilus") refers to extracellular structures of bacterial cells composed of protein monomers (*e.g.*, pilin monomers) which are organized into ordered and repetitive patterns. Further, pili are structures which are involved in processes such as the attachment of bacterial cells to host cell surface receptors, inter-cellular genetic exchanges, and cell-cell recognition. Examples of pili include Type-1 pili, P-pili, F1C pili, S-pili, and 987P-pili. Additional examples of pili are set out below.
- [0089] Pilus-like structure: As used herein, the phrase "pilus-like structure" refers to structures having characteristics similar to that of pili and composed of protein monomers. One example of a "pilus-like structure" is a structure formed by a bacterial cell which expresses modified pilin proteins that do not form ordered and repetitive arrays that are essentially identical to those of natural pili.\
- [0090] Purified: As used herein, when the term "purified" is used in reference to a molecule, it means that the concentration of the molecule being purified has been increased relative to molecules associated with it in its natural environment. Naturally associated molecules include proteins, nucleic acids, lipids and sugars but generally do not include water, buffers, and reagents added to maintain the integrity or facilitate the purification of the molecule being purified. For example, even if mRNA is diluted with an aqueous solvent during oligo dT column chromatography, mRNA molecules are purified by this chromatography if naturally associated nucleic acids and other biological molecules do not bind to the column and are separated from the subject mRNA molecules.
- [0091] Receptor: As used herein, the term "receptor" refers to proteins or glycoproteins or fragments thereof capable of interacting with another molecule, called the ligand. The ligand may belong to any class of biochemical or chemical compounds. The receptor need not necessarily be a membrane-bound protein. Soluble protein, like *e.g.*, maltose binding protein or retinol binding protein are receptors as well.
- [0092] Residue: As used herein, the term "residue" is meant to mean a specific amino acid in a polypeptide backbone or side chain.

- [0093] Temperature-sensitive: As used herein, the phrase "temperature-sensitive" refers to an enzyme which readily catalyzes a reaction at one temperature but catalyzes the same reaction slowly or not at all at another temperature. An example of a temperature-sensitive enzyme is the replicase protein encoded by the pCYTts vector, which has readily detectable replicase activity at temperatures below 34°C and has low or undetectable activity at 37°C.
- [0094] Transcription: As used herein, the term "transcription" refers to the production of RNA molecules from DNA templates catalyzed by RNA polymerase.
- [0095] Recombinant host cell: As used herein, the term "recombinant host cell" refers to a host cell into which one or more nucleic acid molecules of the invention have been introduced.
- [0096] Recombinant virus: As used herein, the phrase "recombinant virus" refers to a virus that is genetically modified by the hand of man. The phrase covers any virus known in the art. More specifically, the phrase refers to an alphavirus genetically modified by the hand of man, and most specifically, the phrase refers to a Sinbis virus genetically modified by the hand of man.
- [0097] Restrictive temperature: As used herein, the phrase "restrictive temperature" refers to temperatures at which an enzyme has low or undetectable levels of catalytic activity. Both "hot" and "cold" sensitive mutants are known and, thus, a restrictive temperature may be higher or lower than a permissive temperature.
- [0098] RNA-dependent RNA replication event: As used herein, the phrase "RNA-dependent RNA replication event" refers to processes which result in the formation of an RNA molecule using an RNA molecule as a template.
- [0099] RNA-Dependent RNA polymerase: As used herein, the phrase "RNA-Dependent RNA polymerase" refers to a polymerase which catalyzes the production of an RNA molecule from another RNA molecule. This term is used herein synonymously with the term "replicase."

[0100] Untranslated RNA: As used herein, the phrase "untranslated RNA" refers to an RNA sequence or molecule which does not encode an open reading frame or encodes an open reading frame, or portion thereof, but in a format in which an amino acid sequence will not be produced (*e.g.*, no initiation codon is present). Examples of such molecules are tRNA molecules, rRNA molecules, and ribozymes.

[0101] Vector: As used herein, the term "vector" refers to an agent (*e.g.*, a plasmid or virus) used to transmit genetic material to a host cell. A vector may be composed of either DNA or RNA.

[0102] one, a, or an: When the terms "one," "a," or "an" are used in this disclosure, they mean "at least one" or "one or more," unless otherwise indicated.

2. Compositions of Ordered and Repetitive Antigen or Antigenic Determinant Arrays and Methods to Make the Same

[0103] The disclosed invention provides compositions comprising an ordered and repetitive antigen or antigenic determinant. Furthermore, the invention conveniently enables the practitioner to construct ordered and repetitive antigen or antigenic determinant arrays for various treatment purposes, which includes the prevention of infectious diseases, the treatment of allergies and the treatment of cancers. The invention also enables the practitioner to construct compositions comprising Pili inducing Th2 immune responses, useful in the treatment of chronic diseases.

[0104] Compositions of the invention essentially comprise, or alternatively consist of, two elements: (1) a non-natural molecular scaffold; and (2) an antigen or antigenic determinant with at least one second attachment site capable of association through at least one non-peptide bond to said first attachment site.

[0105] The non-natural molecular scaffold comprises, or alternatively consists of: (a) a core particle selected from the group consisting of (1) a core particle of non-natural origin and (2) a core particle of natural origin; and (b) an organizer

comprising at least one first attachment site, wherein said organizer is connected to said core particle by at least one covalent bond.

[0106] Compositions of the invention also comprise, or alternatively consist of, core particles to which antigens or antigenic determinants are directly linked.

[0107] The antigen or antigenic determinant has at least one second attachment site which is selected from the group consisting of (a) an attachment site not naturally occurring with said antigen or antigenic determinant; and (b) an attachment site naturally occurring with said antigen or antigenic determinant.

[0108] The invention provides for an ordered and repetitive antigen array through an association of the second attachment site to the first attachment site by way of at least one non-peptide bond. Thus, the antigen or antigenic determinant and the non-natural molecular scaffold are brought together through this association of the first and the second attachment site to form an ordered and repetitive antigen array.

[0109] The practitioner may specifically design the antigen or antigenic determinant and the second attachment site such that the arrangement of all the antigens or antigenic determinants bound to the non-natural molecular scaffold or, in certain embodiments, the core particle will be uniform. For example, one may place a single second attachment site on the antigen or antigenic determinant at the carboxyl or amino terminus, thereby ensuring through design that all antigen or antigenic determinant molecules that are attached to the non-natural molecular scaffold are positioned in a uniform way. Thus, the invention provides a convenient means of placing any antigen or antigenic determinant onto a non-natural molecular scaffold in a defined order and in a manner which forms a repetitive pattern.

[0110] As will be clear to those skilled in the art, certain embodiments of the invention involve the use of recombinant nucleic acid technologies such as cloning, polymerase chain reaction, the purification of DNA and RNA, the expression of recombinant proteins in prokaryotic and eukaryotic cells, etc. Such methodologies are well known to those skilled in the art and may be conveniently

found in published laboratory methods manuals (*e.g.*, Sambrook, J. *et al.*, eds., MOLECULAR CLONING, A LABORATORY MANUAL, 2nd. edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); Ausubel, F. *et al.*, eds., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John H. Wiley & Sons, Inc. (1997)). Fundamental laboratory techniques for working with tissue culture cell lines (Celis, J., ed., CELL BIOLOGY, Academic Press, 2<sup>nd</sup> edition, (1998)) and antibody-based technologies (Harlow, E. and Lane, D., "Antibodies: A Laboratory Manual," Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1988); Deutscher, M.P., "Guide to Protein Purification," *Meth. Enzymol.* 128, Academic Press San Diego (1990); Scopes, R.K., "Protein Purification Principles and Practice," 3<sup>rd</sup> ed., Springer-Verlag, New York (1994)) are also adequately described in the literature, all of which are incorporated herein by reference.

#### A. Construction of a Non-Natural Molecular Scaffold

[0111] One element in compositions of the invention is a non-natural molecular scaffold comprising, or alternatively consisting of, a core particle and an organizer. As used herein, the phrase "non-natural molecular scaffold" refers to any product made by the hand of man that may serve to provide a rigid and repetitive array of first attachment sites. More specifically, the non-natural molecular scaffold comprises, or alternatively consists of, (a) a core particle selected from the group consisting of (1) a core particle of non-natural origin and (2) a core particle of natural origin; and (b) an organizer comprising at least one first attachment site, wherein said organizer is connected to said core particle by at least one covalent bond.

[0112] As will be readily apparent to those skilled in the art, the core particle of the non-natural molecular scaffold of the invention is not limited to any specific form. The core particle may be organic or non-organic and may be synthesized chemically or through a biological process.

[0113] In one embodiment, a non-natural core particle may be a synthetic polymer, a lipid micelle or a metal. Such core particles are known in the art, providing a basis from which to build the novel non-natural molecular scaffold of the invention. By way of example, synthetic polymer or metal core particles are described in U.S. Patent No. 5,770,380, which discloses the use of a calixarene organic scaffold to which is attached a plurality of peptide loops in the creation of an 'antibody mimic', and U.S. Patent No. 5,334,394 describes nanocrystalline particles used as a viral decoy that are composed of a wide variety of inorganic materials, including metals or ceramics. Suitable metals include chromium, rubidium, iron, zinc, selenium, nickel, gold, silver, platinum. Suitable ceramic materials in this embodiment include silicon dioxide, titanium dioxide, aluminum oxide, ruthenium oxide and tin oxide. The core particles of this embodiment may be made from organic materials including carbon (diamond). Suitable polymers include polystyrene, nylon and nitrocellulose. For this type of nanocrystalline particle, particles made from tin oxide, titanium dioxide or carbon (diamond) are may also be used. A lipid micelle may be prepared by any means known in the art. For example micelles may be prepared according to the procedure of Baiselle and Millar (*Biophys. Chem.* 4:355-361 (1975)) or Corti *et al.* (*Chem. Phys. Lipids* 38:197-214 (1981)) or Lopez *et al.* (*FEBS Lett.* 426:314-318 (1998)) or Topchieva and Karezin (*J. Colloid Interface Sci.* 213:29-35 (1999)) or Morein *et al.*, (*Nature* 308:457-460 (1984)), which are all incorporated herein by reference.

[0114] The core particle may also be produced through a biological process, which may be natural or non-natural. By way of example, this type of embodiment may includes a core particle comprising, or alternatively consisting of, a virus, virus-like particle, a bacterial pilus, a phage, a viral capsid particle or a recombinant form thereof. In a more specific embodiment, the core particle may comprise, or alternatively consist of, recombinant proteins of Rotavirus, recombinant proteins of Norwalk virus, recombinant proteins of Alphavirus, recombinant proteins which form bacterial pili or pilus-like structures, recombinant proteins of Foot and Mouth Disease virus, recombinant proteins of

Retrovirus, recombinant proteins of Hepatitis B virus (*e.g.*, a HBcAg), recombinant proteins of Tobacco mosaic virus, recombinant proteins of Flock House Virus, and recombinant proteins of human Papillomavirus.

[0115] Whether natural or non-natural, the core particle of the invention will generally have an organizer that is attached to the natural or non-natural core particle by at least one covalent bond. The organizer is an element bound to a core particle in a non-random fashion that provides a nucleation site for creating an ordered and repetitive antigen array. Ideally, but not necessarily, the organizer is associated with the core particle in a geometric order. Minimally, the organizer comprises a first attachment site.

[0116] In some embodiments of the invention, the ordered and repetitive array is formed by association between (1) either core particles or non-natural molecular scaffolds and (2) an antigen or antigenic determinant. For example, bacterial pili or pilus-like structures are formed from proteins which are organized into ordered and repetitive structures. Thus, in many instances, it will be possible to form ordered arrays of antigens or antigenic determinants by linking these constituents to bacterial pili or pili-like structures.

[0117] As previously stated, the organizer may be any element comprising at least one first attachment site that is bound to a core particle by at least one covalent bond. An organizer may be a protein, a polypeptide, a peptide, an amino acid (*i.e.*, a residue of a protein, a polypeptide or peptide), a sugar, a polynucleotide, a natural or synthetic polymer, a secondary metabolite or compound (biotin, fluorescein, retinol, digoxigenin, metal ions, phenylmethylsulfonylfluoride), or a combination thereof, or a chemically reactive group thereof. In a more specific embodiment, the organizer may comprise a first attachment site comprising an antigen, an antibody or antibody fragment, biotin, avidin, strepavidin, a receptor, a receptor ligand, a ligand, a ligand-binding protein, an interacting leucine zipper polypeptide, an amino group, a chemical group reactive to an amino group; a carboxyl group, chemical group reactive to a carboxyl group, a sulfhydryl group, a chemical group reactive to a sulfhydryl group, or a combination thereof.



[0118] In one embodiment, the core particle of the non-natural molecular scaffold comprises a virus, a bacterial pilus, a structure formed from bacterial pilin, a bacteriophage, a virus-like particle, a viral capsid particle or a recombinant form thereof. Any virus known in the art having an ordered and repetitive coat and/or core protein structure may be selected as a non-natural molecular scaffold of the invention; examples of suitable viruses include: sindbis and other alphaviruses; vesicular stomatitis virus; rhabdo-, (e.g. vesicular stomatitis virus), picorna-, toga-, orthomyxo-, polyoma-, parvovirus, rotavirus, Norwalk virus, foot and mouth disease virus, a retrovirus, Hepatitis B virus, Tobacco mosaic virus, flock house virus, human papillomavirus (for example, see Table 1 in Bachman, M.F. and Zinkernagel, R.M., *Immunol. Today* 17:553-558 (1996)).

[0119] In one embodiment, the invention utilizes genetic engineering of a virus to create a fusion between an ordered and repetitive viral envelope protein and an organizer comprising a heterologous protein, peptide, antigenic determinant or a reactive amino acid residue of choice. Other genetic manipulations known to those in the art may be included in the construction of the non-natural molecular scaffold; for example, it may be desirable to restrict the replication ability of the recombinant virus through genetic mutation. The viral protein selected for fusion to the organizer (*i.e.*, first attachment site) protein should have an organized and repetitive structure. Such an organized and repetitive structure include paracrystalline organizations with a spacing of 5-15 nm on the surface of the virus. The creation of this type of fusion protein will result in multiple, ordered and repetitive organizers on the surface of the virus. Thus, the ordered and repetitive organization of the first attachment sites resulting therefrom will reflect the normal organization of the native viral protein.

[0120] As will be discussed in more detail herein, in another embodiment of the invention, the non-natural molecular scaffold is a recombinant alphavirus, and more specifically, a recombinant Sinbis virus. Alphaviruses are positive stranded RNA viruses that replicate their genomic RNA entirely in the cytoplasm of the infected cell and without a DNA intermediate (Strauss, J. and Strauss, E.,

*Microbiol. Rev.* 58:491-562 (1994)). Several members of the alphavirus family, Sindbis (Xiong, C. *et al.*, *Science* 243:1188-1191 (1989); Schlesinger, S., *Trends Biotechnol.* 11:18-22 (1993)), Semliki Forest Virus (SFV) (Liljeström, P. & Garoff, H., *Bio/Technology* 9:1356-1361 (1991)) and others (Davis, N.L. *et al.*, *Virology* 171:189-204 (1989)), have received considerable attention for use as virus-based expression vectors for a variety of different proteins (Lundstrom, K., *Curr. Opin. Biotechnol.* 8:578-582 (1997); Liljeström, P., *Curr. Opin. Biotechnol.* 5:495-500 (1994)) and as candidates for vaccine development. Recently, a number of patents have issued directed to the use of alphaviruses for the expression of heterologous proteins and the development of vaccines (see U.S. Patent Nos. 5,766,602; 5,792,462; 5,739,026; 5,789,245 and 5,814,482). The construction of the alphaviral scaffold of the invention may be done by means generally known in the art of recombinant DNA technology, as described by the aforementioned articles, which are incorporated herein by reference.

[0121] A variety of different recombinant host cells can be utilized to produce a viral-based core particle for antigen or antigenic determinant attachment. For example, Alphaviruses are known to have a wide host range; Sindbis virus infects cultured mammalian, reptilian, and amphibian cells, as well as some insect cells (Clark, H., *J. Natl. Cancer Inst.* 51:645 (1973); Leake, C., *J. Gen. Virol.* 35:335 (1977); Stollar, V. in *THE TOGAVIRUSES*, R.W. Schlesinger, Ed., Academic Press, (1980), pp.583-621). Thus, numerous recombinant host cells can be used in the practice of the invention. BHK, COS, Vero, HeLa and CHO cells are particularly suitable for the production of heterologous proteins because they have the potential to glycosylate heterologous proteins in a manner similar to human cells (Watson, E. *et al.*, *Glycobiology* 4:227, (1994)) and can be selected (Zang, M. *et al.*, *Bio/Technology* 13:389 (1995)) or genetically engineered (Renner W. *et al.*, *Biotech. Bioeng.* 4:476 (1995); Lee K. *et al. Biotech. Bioeng.* 50:336 (1996)) to grow in serum-free medium, as well as in suspension.

[0122] Introduction of the polynucleotide vectors into host cells can be effected by methods described in standard laboratory manuals (see, e.g., Sambrook, J. *et*

*al.*, eds., MOLECULAR CLONING, A LABORATORY MANUAL, 2nd. edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989), Chapter 9; Ausubel, F. *et al.*, eds., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John H. Wiley & Sons, Inc. (1997), Chapter 16), including methods such as electroporation, DEAE-dextran mediated transfection, transfection, microinjection, cationic lipid-mediated transfection, transduction, scrape loading, ballistic introduction, and infection. Methods for the introduction of exogenous DNA sequences into host cells are discussed in Felgner, P. *et al.*, U.S. Patent No. 5,580,859.

[0123] Packaged RNA sequences can also be used to infect host cells. These packaged RNA sequences can be introduced to host cells by adding them to the culture medium. For example, the preparation of non-infective alphaviral particles is described in a number of sources, including "Sindbis Expression System", Version C (*Invitrogen* Catalog No. K750-1).

[0124] When mammalian cells are used as recombinant host cells for the production of viral-based core particles, these cells will generally be grown in tissue culture. Methods for growing cells in culture are well known in the art (*see, e.g.*, Celis, J., ed., CELL BIOLOGY, Academic Press, 2<sup>nd</sup> edition, (1998); Sambrook, J. *et al.*, eds., MOLECULAR CLONING, A LABORATORY MANUAL, 2nd. edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); Ausubel, F. *et al.*, eds., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John H. Wiley & Sons, Inc. (1997); Freshney, R., CULTURE OF ANIMAL CELLS, Alan R. Liss, Inc. (1983)).

[0125] As will be understood by those in the art, the first attachment site may be or be a part of any suitable protein, polypeptide, sugar, polynucleotide, peptide (amino acid), natural or synthetic polymer, a secondary metabolite or combination thereof that may serve to specifically attach the antigen or antigenic determinant of choice to the non-natural molecular scaffold. In one embodiment, the attachment site is a protein or peptide that may be selected from those known in the art. For example, the first attachment site may be selected from the following

group: a ligand, a receptor, a lectin, avidin, streptavidin, biotin, an epitope such as an HA or T7 tag, Myc, Max, immunoglobulin domains and any other amino acid sequence known in the art that would be useful as a first attachment site.

[0126] It should be further understood by those in the art that with another embodiment of the invention, the first attachment site may be created secondarily to the organizer (*i.e.*, protein or polypeptide) utilized in constructing the in-frame fusion to the capsid protein. For example, a protein may be utilized for fusion to the envelope protein with an amino acid sequence known to be glycosylated in a specific fashion, and the sugar moiety added as a result may then serve at the first attachment site of the viral scaffold by way of binding to a lectin serving as the secondary attachment site of an antigen. Alternatively, the organizer sequence may be biotinylated *in vivo* and the biotin moiety may serve as the first attachment site of the invention, or the organizer sequence may be subjected to chemical modification of distinct amino acid residues *in vitro*, the modification serving as the first attachment site.

[0127] One specific embodiment of the invention utilizes the Sinbis virus. The Sinbis virus RNA genome is packaged into a capsid protein that is surrounded by a lipid bilayer containing three proteins called E1, E2, and E3. These so-called envelope proteins are glycoproteins, and the glycosylated portions are located on the outside of the lipid bilayer, where complexes of these proteins form the "spikes" that can be seen in electron micrographs to project outward from the surface of the virus. In another embodiment of the invention, the first attachment site is selected to be the *JUN* or *FOS* leucine zipper protein domain that is fused in frame to the E2 envelope protein. However, it will be clear to all individuals in the art that other envelope proteins may be utilized in the fusion protein construct for locating the first attachment site in the non-natural molecular scaffold of the invention.

[0128] In a specific embodiment of the invention, the first attachment site is selected to be the *JUN-FOS* leucine zipper protein domain that is fused in frame to the Hepatitis B capsid (core) protein (HBcAg). However, it will be clear to all

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individuals in the art that other viral capsid proteins may be utilized in the fusion protein construct for locating the first attachment site in the non-natural molecular scaffold of the invention.

[0129] In another specific embodiment of the invention, the first attachment site is selected to be a lysine or cysteine residue that is fused in frame to the HBcAg. However, it will be clear to all individuals in the art that other viral capsid or virus-like particles may be utilized in the fusion protein construct for locating the first attachment in the non-natural molecular scaffold of the invention.

[0130] Example 1 is provided to demonstrate the construction of an in-frame fusion protein between the Sinbis virus E2 envelope protein and the *JUN* leucine zipper protein domain using the pTE5'2J vector of Hahn *et al.* (*Proc. Natl. Acad. Sci. USA* 89:2679-2683 (1992)). The *JUN* amino acid sequence utilized for the first attachment site is the following: CGGRIARLEEKVKTLKAQNSE LASTANMLREQVAQLKQKVMNHVGC (SEQ ID NO:59). In this instance, the anticipated second attachment site on the antigen would be the *FOS* leucine zipper protein domain and the amino acid sequence would be the following: CGGLTDTLQAETDQVEDEKSALQTEIANLLKEKEKLEFILAAHGGC (SEQ ID NO:60)

[0131] These sequences are derived from the transcription factors *JUN* and *FOS*, each flanked with a short sequence containing a cysteine residue on both sides. These sequences are known to interact with each other. The original hypothetical structure proposed for the *JUN-FOS* dimer assumed that the hydrophobic side chains of one monomer interdigitate with the respective side chains of the other monomer in a zipper-like manner (Landschulz *et al.*, *Science* 240:1759-1764 (1988)). However, this hypothesis proved to be wrong, and these proteins are known to form an  $\alpha$ -helical coiled coil (O'Shea *et al.*, *Science* 243:538-542 (1989); O'Shea *et al.*, *Cell* 68:699-708 (1992); Cohen & Parry, *Trends Biochem. Sci.* 11:245-248 (1986)). Thus, the term "leucine zipper" is frequently used to refer to these protein domains for more historical than structural reasons. Throughout this patent, the term "leucine zipper" is used to refer to the sequences

depicted above or sequences essentially similar to the ones depicted above. The terms *JUN* and *FOS* are used for the respective leucine zipper domains rather than the entire *JUN* and *FOS* proteins.

[0132] In one embodiment, the invention provides for the production of a Sinbis virus E2-*JUN* scaffold using the pCYTts expression system (WO 99/50432). The pCYTts expression system provides novel expression vectors which permit tight regulation of gene expression in eucaryotic cells. The DNA vectors of this system are transcribed to form RNA molecules which are then replicated by a temperature-sensitive replicase to form additional RNA molecules. The RNA molecules produced by replication contain a nucleotide sequence which may be translated to produce a protein of interest or which encode one or more untranslated RNA molecules. Thus the expression system enables the production of recombinant Sinbis virus particles.

[0133] Example 2 provides details on the production of the E2-*JUN* Sinbis non-natural molecular scaffold of the invention. Additionally provided in Example 3 is another method for the production of recombinant E2-*JUN* Sinbis virus scaffold using the pTE5'2JE2:*JUN* vector produced in Example 1. Thus the invention provides two means, the pCYTts expression system (Example 2) and the pTE5'2J vector system (Example 3) by which recombinant Sinbis virus E2-*JUN* non-natural molecular scaffold may be produced. An analysis of viral particles produced in each system is provided in Figure 1 and Figure 2.

[0134] As previously stated, the invention includes viral-based core particles which comprise, or alternatively consist of, a virus, virus-like particle, a phage, a viral capsid particle or a recombinant form thereof. Skilled artisans have the knowledge to produce such core particles and attach organizers thereto. By way of providing other examples, the invention provides herein for the production of Hepatitis B virus-like particles and measles viral capsid particles as core particles (Examples 17 to 22). In such an embodiment, the *JUN* leucine zipper protein domain or *FOS* leucine zipper protein domain may be used as an organizer, and

hence as a first attachment site, for the non-natural molecular scaffold of the invention.

[0135] Examples 23-29 provide details of the production of Hepatitis B core particles carrying an in-frame fused peptide with a reactive lysine residue and antigens carrying a genetically fused cysteine residue, as first and second attachment site, respectively.

[0136] In other embodiments, the core particles used in compositions of the invention are composed of a Hepatitis B capsid (core) protein (HBcAg), or fragment thereof, which has been modified to either eliminate or reduce the number of free cysteine residues. Zhou *et al.* (*J. Virol.* 66:5393-5398 (1992)) demonstrated that HBcAgs which have been modified to remove the naturally resident cysteine residues retain the ability to associate and form multimeric structures. Thus, core particles suitable for use in compositions of the invention include those comprising modified HBcAgs, or fragments thereof, in which one or more of the naturally resident cysteine residues have been either deleted or substituted with another amino acid residue (*e.g.*, a serine residue).

[0137] The HBcAg is a protein generated by the processing of a Hepatitis B core antigen precursor protein. A number of isotypes of the HBcAg have been identified. For example, the HBcAg protein having the amino acid sequence shown in SEQ ID NO:132 is 183 amino acids in length and is generated by the processing of a 212 amino acid Hepatitis B core antigen precursor protein. This processing results in the removal of 29 amino acids from the N-terminus of the Hepatitis B core antigen precursor protein. Similarly, the HBcAg protein having the amino acid sequence shown in SEQ ID NO:134 is 185 amino acids in length and is generated by the processing of a 214 amino acid Hepatitis B core antigen precursor protein. The amino acid sequence shown in SEQ ID NO:134, as compared to the amino acid sequence shown in SEQ ID NO:132, contains a two amino acid insert at positions 152 and 153 in SEQ ID NO:134.

[0138] In most instances, vaccine compositions of the invention will be prepared using the processed form of a HBcAg (*i.e.*, a HBcAg from which the N-terminal

leader sequence (*e.g.*, the first 29 amino acid residues shown in SEQ ID NO:134) of the Hepatitis B core antigen precursor protein have been removed).

[0139] Further, when HBcAgs are produced under conditions where processing will not occur, the HBcAgs will generally be expressed in "processed" form. For example, bacterial systems, such as *E. coli*, generally do not remove the leader sequences of proteins which are normally expressed in eukaryotic cells. Thus, when an *E. coli* expression system is used to produce HBcAgs of the invention, these proteins will generally be expressed such that the N-terminal leader sequence of the Hepatitis B core antigen precursor protein is not present.

[0140] In one embodiment of the invention, a modified HBcAg comprising the amino acid sequence shown in SEQ ID NO:134, or subportion thereof, is used to prepare non-natural molecular scaffolds. In particular, modified HBcAgs suitable for use in the practice of the invention include proteins in which one or more of the cysteine residues at positions corresponding to positions 48, 61, 107 and 185 of a protein having the amino acid sequence shown in SEQ ID NO:134 have been either deleted or substituted with other amino acid residues (*e.g.*, a serine residue). As one skilled in the art would recognize, cysteine residues at similar locations in HBcAg variants having amino acids sequences which differ from that shown in SEQ ID NO:134 could also be deleted or substituted with other amino acid residues. The modified HBcAg variants can then be used to prepare vaccine compositions of the invention.

[0141] The present invention also includes HBcAg variants which have been modified to delete or substitute one or more additional cysteine residues which are not found in polypeptides having the amino acid sequence shown in SEQ ID NO:134. Examples of such HBcAg variants have the amino acid sequences shown in SEQ ID NOs:90 and 132. These variant contain cysteines residues at a position corresponding to amino acid residue 147 in SEQ ID NO:134. Thus, the vaccine compositions of the invention include compositions comprising HBcAgs in which cysteine residues not present in the amino acid sequence shown in SEQ ID NO:134 have been deleted.



[0142] Under certain circumstances (*e.g.*, when a heterobifunctional cross-linking reagent is used to attach antigens or antigenic determinants to the non-natural molecular scaffold), the presence of free cysteine residues in the HBcAg is believed to lead to covalent coupling of toxic components to core particles, as well as the cross-linking of monomers to form undefined species.

[0143] Further, in many instances, these toxic components may not be detectable with assays performed on compositions of the invention. This is so because covalent coupling of toxic components to the non-natural molecular scaffold would result in the formation of a population of diverse species in which toxic components are linked to different cysteine residues, or in some cases no cysteine residues, of the HBcAgs. In other words, each free cysteine residue of each HBcAg will not be covalently linked to toxic components. Further, in many instances, none of the cysteine residues of particular HBcAgs will be linked to toxic components. Thus, the presence of these toxic components may be difficult to detect because they would be present in a mixed population of molecules. The administration to an individual of HBcAg species containing toxic components, however, could lead to a potentially serious adverse reaction.

[0144] It is well known in the art that free cysteine residues can be involved in a number of chemical side reactions. These side reactions include disulfide exchanges, reaction with chemical substances or metabolites that are, for example, injected or formed in a combination therapy with other substances, or direct oxidation and reaction with nucleotides upon exposure to UV light. Toxic adducts could thus be generated, especially considering the fact that HBcAgs have a strong tendency to bind nucleic acids. Detection of such toxic products in antigen-capsid conjugates would be difficult using capsids prepared using HBcAgs containing free cysteines and heterobifunctional cross-linkers, since a distribution of products with a broad range of molecular weight would be generated. The toxic adducts would thus be distributed between a multiplicity of species, which individually may each be present at low concentration, but reach toxic levels when together.

[0145] In view of the above, one advantage to the use of HBcAgs in vaccine compositions which have been modified to remove naturally resident cysteine residues is that sites to which toxic species can bind when antigens or antigenic determinants are attached to the non-natural molecular scaffold would be reduced in number or eliminated altogether. Further, a high concentration of cross-linker can be used to produce highly decorated particles without the drawback of generating a plurality of undefined cross-linked species of HBcAg monomers (*i.e.*, a diverse mixture of cross-linked monomeric HbcAgs).

[0146] A number of naturally occurring HBcAg variants suitable for use in the practice of the present invention have been identified. Yuan *et al.*, (*J. Virol.* 73:10122-10128 (1999)), for example, describe variants in which the isoleucine residue at position corresponding to position 97 in SEQ ID NO:134 is replaced with either a leucine residue or a phenylalanine residue. The amino acid sequences of a number of HBcAg variants, as well as several Hepatitis B core antigen precursor variants, are disclosed in GenBank reports AAF121240 (SEQ ID NO:89), AF121239 (SEQ ID NO:90), X85297 (SEQ ID NO:91), X02496 (SEQ ID NO:92), X85305 (SEQ ID NO:93), X85303 (SEQ ID NO:94), AF151735 (SEQ ID NO:95), X85259 (SEQ ID NO:96), X85286 (SEQ ID NO:97), X85260 (SEQ ID NO:98), X85317 (SEQ ID NO:99), X85298 (SEQ ID NO:100), AF043593 (SEQ ID NO:101), M20706 (SEQ ID NO:102), X85295 (SEQ ID NO:103), X80925 (SEQ ID NO:104), X85284 (SEQ ID NO:105), X85275 (SEQ ID NO:106), X72702 (SEQ ID NO:107), X85291 (SEQ ID NO:108), X65258 (SEQ ID NO:109), X85302 (SEQ ID NO:110), M32138 (SEQ ID NO:111), X85293 (SEQ ID NO:112), X85315 (SEQ ID NO:113), U95551 (SEQ ID NO:114), X85256 (SEQ ID NO:115), X85316 (SEQ ID NO:116), X85296 (SEQ ID NO:117), AB033559 (SEQ ID NO:118), X59795 (SEQ ID NO:119), X85299 (SEQ ID NO:120), X85307 (SEQ ID NO:121), X65257 (SEQ ID NO:122), X85311 (SEQ ID NO:123), X85301 (SEQ ID NO:124), X85314 (SEQ ID NO:125), X85287 (SEQ ID NO:126), X85272 (SEQ ID NO:127), X85319 (SEQ ID NO:128), AB010289 (SEQ ID NO:129), X85285 (SEQ ID NO:130),

AB010289 (SEQ ID NO:131), AF121242 (SEQ ID NO:132), M90520 (SEQ ID NO:135), P03153 (SEQ ID NO:136), AF110999 (SEQ ID NO:137), and M95589 (SEQ ID NO:138), the disclosures of each of which are incorporated herein by reference. These HBcAg variants differ in amino acid sequence at a number of positions, including amino acid residues which corresponds to the amino acid residues located at positions 12, 13, 21, 22, 24, 29, 32, 33, 35, 38, 40, 42, 44, 45, 49, 51, 57, 58, 59, 64, 66, 67, 69, 74, 77, 80, 81, 87, 92, 93, 97, 98, 100, 103, 105, 106, 109, 113, 116, 121, 126, 130, 133, 135, 141, 147, 149, 157, 176, 178, 182 and 183 in SEQ ID NO:134. The invention is also directed to amino acid sequences that are at least 65, 70, 75, 80, 85, 90 or 95% identical to the above Hepatitis B viral capsid protein sequences. HBcAgs suitable for use in the present invention may be derived from any organism so long as they are able to associate to form an ordered and repetitive antigen array.

[0147] As noted above, generally processed HBcAgs (*i.e.*, those which lack leader sequences) will be used in the vaccine compositions of the invention. Thus, when HBcAgs having amino acid sequence shown in SEQ ID NOs:136, 137, or 138 are used to prepare vaccine compositions of the invention, generally 30, 35-43, or 35-43 amino acid residues at the N-terminus, respectively, of each of these proteins will be omitted.

[0148] The present invention includes vaccine compositions, as well as methods for using these compositions, which employ the above described variant HBcAgs for the preparation of non-natural molecular scaffolds.

[0149] Further included within the scope of the invention are additional HBcAg variants which are capable of associating to form dimeric or multimeric structures. Thus, the invention further includes vaccine compositions comprising HBcAg polypeptides comprising, or alternatively consisting of, amino acid sequences which are at least 80%, 85%, 90%, 95%, 97%, or 99% identical to any of the amino acid sequences shown in SEQ ID NOs:89-132 and 134-138, and forms of these proteins which have been processed, where appropriate, to remove the N-terminal leader sequence.

[0150] Whether the amino acid sequence of a polypeptide has an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97%, or 99% identical to one of the amino acid sequences shown in SEQ ID NOs:89-132 and 134-138, or a subportion thereof, can be determined conventionally using known computer programs such the Bestfit program. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference amino acid sequence according to the present invention, the parameters are set such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

[0151] The HBcAg variants and precursors having the amino acid sequences set out in SEQ ID NOs:89-132 and 134-136 are relatively similar to each other. Thus, reference to an amino acid residue of a HBcAg variant located at a position which corresponds to a particular position in SEQ ID NO:134, refers to the amino acid residue which is present at that position in the amino acid sequence shown in SEQ ID NO:134. The homology between these HBcAg variants is for the most part high enough among Hepatitis B viruses that infect mammals so that one skilled in the art would have little difficulty reviewing both the amino acid sequence shown in SEQ ID NO:134 and that of a particular HBcAg variant and identifying "corresponding" amino acid residues. For example, the HBcAg amino acid sequence shown in SEQ ID NO:135, which shows the amino acid sequence of a HBcAg derived from a virus which infect woodchucks, has enough homology to the HBcAg having the amino acid sequence shown in SEQ ID NO:134 that it is readily apparent that a three amino acid residue insert is present in SEQ ID NO:135 between amino acid residues 155 and 156 of SEQ ID NO:134.

[0152] The HBcAgs of Hepatitis B viruses which infect snow geese and ducks differ enough from the amino acid sequences of HBcAgs of Hepatitis B viruses which infect mammals that alignment of these forms of this protein with the amino acid sequence shown in SEQ ID NO:134 is difficult. However, the invention

includes vaccine compositions which comprise HBcAg variants of Hepatitis B viruses which infect birds, as well as vaccine compositions which comprise fragments of these HBcAg variants. HBcAg fragments suitable for use in preparing vaccine compositions of the invention include compositions which contain polypeptide fragments comprising, or alternatively consisting of, amino acid residues selected from the group consisting of 36-240, 36-269, 44-240, 44-269, 36-305, and 44-305 of SEQ ID NO:137 or 36-240, 36-269, 44-240, 44-269, 36-305, and 44-305 of SEQ ID NO:138. As one skilled in the art would recognize, one, two, three or more of the cysteine residues naturally present in these polypeptides (*e.g.*, the cysteine residues at position 153 in SEQ ID NO:137 or positions 34, 43, and 196 in SEQ ID NO:138) could be either substituted with another amino acid residue or deleted prior to their inclusion in vaccine compositions of the invention.

[0153] In one embodiment, the cysteine residues at positions 48 and 107 of a protein having the amino acid sequence shown in SEQ ID NO:134 are deleted or substituted with another amino acid residue but the cysteine at position 61 is left in place. Further, the modified polypeptide is then used to prepare vaccine compositions of the invention.

[0154] As set out below in Example 31, the cysteine residues at positions 48 and 107, which are accessible to solvent, may be removed, for example, by site-directed mutagenesis. Further, the inventors have found that the Cys-48-Ser, Cys-107-Ser HBcAg double mutant constructed as described in Example 31 can be expressed in *E. coli*.

[0155] As discussed above, the elimination of free cysteine residues reduces the number of sites where toxic components can bind to the HBcAg, and also eliminates sites where cross-linking of lysine and cysteine residues of the same or of neighboring HBcAg molecules can occur. The cysteine at position 61, which is involved in dimer formation and forms a disulfide bridge with the cysteine at position 61 of another HBcAg, will normally be left intact for stabilization of HBcAg dimers and multimers of the invention.

- [0156] As shown in Example 32, cross-linking experiments performed with (1) HBcAgs containing free cysteine residues and (2) HBcAgs whose free cysteine residues have been made unreactive with iodacetamide, indicate that free cysteine residues of the HBcAg are responsible for cross-linking between HBcAgs through reactions between heterobifunctional cross-linker derivatized lysine side chains, and free cysteine residues. Example 32 also indicates that cross-linking of HBcAg subunits leads to the formation of high molecular weight species of undefined size which cannot be resolved by SDS-polyacrylamide gel electrophoresis.
- [0157] When an antigen or antigenic determinant is linked to the non-natural molecular scaffold through a lysine residue, it may be advantageous to either substitute or delete one or both of the naturally resident lysine residues located at positions corresponding to positions 7 and 96 in SEQ ID NO:134, as well as other lysine residues present in HBcAg variants. The elimination of these lysine residues results in the removal of binding sites for antigens or antigenic determinants which could disrupt the ordered array and should improve the quality and uniformity of the final vaccine composition.
- [0158] In many instances, when both of the naturally resident lysine residues at positions corresponding to positions 7 and 96 in SEQ ID NO:134 are eliminated, another lysine will be introduced into the HBcAg as an attachment site for an antigen or antigenic determinant. Methods for inserting such a lysine residue are set out, for example, in Example 23 below. It will often be advantageous to introduce a lysine residue into the HBcAg when, for example, both of the naturally resident lysine residues at positions corresponding to positions 7 and 96 in SEQ ID NO:134 are altered and one seeks to attach the antigen or antigenic determinant to the non-natural molecular scaffold using a heterobifunctional cross-linking agent.
- [0159] The C-terminus of the HBcAg has been shown to direct nuclear localization of this protein. (Eckhardt *et al.*, *J. Virol.* 65:575-582 (1991).) Further, this region of the protein is also believed to confer upon the HBcAg the ability to bind nucleic acids.

[0160] In some embodiments, vaccine compositions of the invention will contain HBcAgs which have nucleic acid binding activity (*e.g.*, which contain a naturally resident HBcAg nucleic acid binding domain). HBcAgs containing one or more nucleic acid binding domains are useful for preparing vaccine compositions which exhibit enhanced T-cell stimulatory activity. Thus, the vaccine compositions of the invention include compositions which contain HBcAgs having nucleic acid binding activity. Further included are vaccine compositions, as well as the use of such compositions in vaccination protocols, where HBcAgs are bound to nucleic acids. These HBcAgs may bind to the nucleic acids prior to administration to an individual or may bind the nucleic acids after administration.

[0161] In other embodiments, vaccine compositions of the invention will contain HBcAgs from which the C-terminal region (*e.g.*, amino acid residues 145-185 or 150-185 of SEQ ID NO:134) has been removed and do not bind nucleic acids. Thus, additional modified HBcAgs suitable for use in the practice of the present invention include C-terminal truncation mutants. Suitable truncation mutants include HBcAgs where 1, 5, 10, 15, 20, 25, 30, 34, 35, 36, 37, 38, 39, 40, 41, 42 or 48 amino acids have been removed from the C-terminus.

[0162] HBcAgs suitable for use in the practice of the present invention also include N-terminal truncation mutants. Suitable truncation mutants include modified HBcAgs where 1, 2, 5, 7, 9, 10, 12, 14, 15, and 17 amino acids have been removed from the N-terminus.

[0163] Further HBcAgs suitable for use in the practice of the present invention include N- and C-terminal truncation mutants. Suitable truncation mutants include HBcAgs where 1, 2, 5, 7, 9, 10, 12, 14, 15, and 17 amino acids have been removed from the N-terminus and 1, 5, 10, 15, 20, 25, 30, 34, 35, 36, 37, 38, 39, 40, 41, 42 or 48 amino acids have been removed from the C-terminus.

[0164] The invention further includes vaccine compositions comprising HBcAg polypeptides comprising, or alternatively consisting of, amino acid sequences which are at least 80%, 85%, 90%, 95%, 97%, or 99% identical to the above described truncation mutants.

[0165] As discussed above, in certain embodiments of the invention, a lysine residue is introduced as a first attachment site into a polypeptide which forms the non-natural molecular scaffold. In preferred embodiments, vaccine compositions of the invention are prepared using a HBcAg comprising, or alternatively consisting of, amino acids 1-144 or amino acids 1-149 of SEQ ID NO:134 which is modified so that the amino acids corresponding to positions 79 and 80 are replaced with a peptide having the amino acid sequence of Gly-Gly-Lys-Gly-Gly (SEQ ID NO:158) and the cysteine residues at positions 48 and 107 are either deleted or substituted with another amino acid residue, while the cysteine at position 61 is left in place. The invention further includes vaccine compositions comprising corresponding fragments of polypeptides having amino acid sequences shown in any of SEQ ID NOs:89-132 and 135-136 which also have the above noted amino acid alterations.

[0166] The invention further includes vaccine compositions comprising fragments of a HBcAg comprising, or alternatively consisting of, an amino acid sequence other than that shown in SEQ ID NO:134 from which a cysteine residue not present at corresponding location in SEQ ID NO:134 has been deleted. One example of such a fragment would be a polypeptide comprising, or alternatively consisting of, amino acids amino acids 1-149 of SEQ ID NO:132 where the cysteine residue at position 147 has been either substituted with another amino acid residue or deleted.

[0167] The invention further includes vaccine compositions comprising HBcAg polypeptides comprising, or alternatively consisting of, amino acid sequences which are at least 80%, 85%, 90%, 95%, 97%, or 99% identical to amino acids 1-144 or 1-149 of SEQ ID NO:134 and corresponding subportions of a polypeptide comprising an amino acid sequence shown in any of SEQ ID NOs:89-132 or 134-136, as well as to amino acids 1-147 or 1-152 of SEQ ID NO:158.

[0168] The invention also includes vaccine compositions comprising HBcAg polypeptides comprising, or alternatively consisting of, amino acid sequences



which are at least 80%, 85%, 90%, 95%, 97%, or 99% identical to amino acids 36-240, 36-269, 44-240, 44-269, 36-305, and 44-305 of SEQ ID NO:137 or 36-240, 36-269, 44-240, 44-269, 36-305, and 44-305 of SEQ ID NO:138.

[0169] Vaccine compositions of the invention may comprise mixtures of different HBcAgs. Thus, these vaccine compositions may be composed of HBcAgs which differ in amino acid sequence. For example, vaccine compositions could be prepared comprising a "wild-type" HBcAg and a modified HBcAg in which one or more amino acid residues have been altered (*e.g.*, deleted, inserted or substituted). In most applications, however, only one type of a HBcAg, or at least HBcAgs having essentially equivalent first attachment sites, will be used because vaccines prepared using such HBcAgs will present highly ordered and repetitive arrays of antigens or antigenic determinants. Further, preferred vaccine compositions of the invention are those which present highly ordered and repetitive antigen arrays.

[0170] The invention further includes vaccine compositions where the non-natural molecular scaffold is prepared using a HBcAg fused to another protein. As discussed above, one example of such a fusion protein is a HBcAg/*FOS* fusion. Other examples of HBcAg fusion proteins suitable for use in vaccine compositions of the invention include fusion proteins where an amino acid sequence has been added which aids in the formation and/or stabilization of HBcAg dimers and multimers. This additional amino acid sequence may be fused to either the N- or C-terminus of the HBcAg. One example, of such a fusion protein is a fusion of a HBcAg with the GCN4 helix region of *Saccharomyces cerevisiae* (GenBank Accession No. P03069 (SEQ ID NO:154)).

[0171] The helix domain of the GCN4 protein forms homodimers via non-covalent interactions which can be used to prepare and stabilize HBcAg dimers and multimers.

[0172] In one embodiment, the invention provides vaccine compositions prepared using HBcAg fusion proteins comprising a HBcAg, or fragment thereof, with a GCN4 polypeptide having the sequence of amino acid residues 227 to 276 in SEQ

ID NO:154 fused to the C-terminus. This GCN4 polypeptide may also be fused to the N-terminus of the HBcAg.

[0173] HBcAg/*src* homology 3 (SH3) domain fusion proteins could also be used to prepare vaccine compositions of the invention. SH3 domains are relatively small domains found in a number of proteins which confer the ability to interact with specific proline-rich sequences in protein binding partners (*see* McPherson, *Cell Signal* 11:229-238 (1999)). HBcAg/SH3 fusion proteins could be used in several ways. First, the SH3 domain could form a first attachment site which interacts with a second attachment site of the antigen or antigenic determinant. Similarly, a proline rich amino acid sequence could be added to the HBcAg and used as a first attachment site for an SH3 domain second attachment site of an antigen or antigenic determinant. Second, the SH3 domain could associate with proline rich regions introduced into HBcAgs. Thus, SH3 domains and proline rich SH3 interaction sites could be inserted into either the same or different HBcAgs and used to form and stabilized dimers and multimers of the invention.

[0174] In other embodiments, a bacterial pilin, a subportion of a bacterial pilin, or a fusion protein which contains either a bacterial pilin or subportion thereof is used to prepare vaccine compositions of the invention. Examples of pilin proteins include pilins produced by *Escherichia coli*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Caulobacter crescentus*, *Pseudomonas stutzeri*, and *Pseudomonas aeruginosa*. The amino acid sequences of pilin proteins suitable for use with the present invention include those set out in GenBank reports AJ000636 (SEQ ID NO:139), AJ132364 (SEQ ID NO:140), AF229646 (SEQ ID NO:141), AF051814 (SEQ ID NO:142), AF051815 (SEQ ID NO:143), and X00981 (SEQ ID NO:155), the entire disclosures of which are incorporated herein by reference.

[0175] Bacterial pilin proteins are generally processed to remove N-terminal leader sequences prior to export of the proteins into the bacterial periplasm. Further, as one skilled in the art would recognize, bacterial pilin proteins used to

prepare vaccine compositions of the invention will generally not have the naturally present leader sequence.

[0176] One specific example of a pilin protein suitable for use in the present invention is the P-pilin of *E. coli* (GenBank report AF237482 (SEQ ID NO:144)). An example of a Type-1 *E. coli* pilin suitable for use with the invention is a pilin having the amino acid sequence set out in GenBank report P04128 (SEQ ID NO:146), which is encoded by nucleic acid having the nucleotide sequence set out in GenBank report M27603 (SEQ ID NO:145). The entire disclosures of these GenBank reports are incorporated herein by reference. Again, the mature form of the above referenced protein would generally be used to prepare vaccine compositions of the invention. Another example of a pilin protein is SEQ ID NO: 184, which is identical to SEQ ID NO: 146, except that in SEQ ID NO: 146, amino acid 20 is threonine, but in SEQ ID NO:184, amino acid 20 is alanine.

[0177] Bacterial pilins or pilin subportions suitable for use in the practice of the present invention will generally be able to associate to form non-natural molecular scaffolds.

[0178] Methods for preparing pili and pilus-like structures *in vitro* are known in the art. Bullitt *et al.*, *Proc. Natl. Acad. Sci. USA* 93:12890-12895 (1996), for example, describe the *in vitro* reconstitution of *E. coli* P-pili subunits. Further, Eshdat *et al.*, *J. Bacteriol.* 148:308-314 (1981) describe methods suitable for dissociating Type-1 pili of *E. coli* and the reconstitution of pili. In brief, these methods are as follows: pili are dissociated by incubation at 37°C in saturated guanidine hydrochloride. Pilin proteins are then purified by chromatography, after which pilin dimers are formed by dialysis against 5 mM tris(hydroxymethyl) aminomethane hydrochloride (pH 8.0). Eshdat *et al.* also found that pilin dimers reassemble to form pili upon dialysis against the 5 mM tris(hydroxymethyl) aminomethane (pH 8.0) containing 5 mM MgCl<sub>2</sub>.

[0179] Further, using, for example, conventional genetic engineering and protein modification methods, pilin proteins may be modified to contain a first attachment site to which an antigen or antigenic determinant is linked through a second

attachment site. Alternatively, antigens or antigenic determinants can be directly linked through a second attachment site to amino acid residues which are naturally resident in these proteins. These modified pilin proteins may then be used in vaccine compositions of the invention.

[0180] Bacterial pilin proteins used to prepare vaccine compositions of the invention may be modified in a manner similar to that described herein for HBcAg. For example, cysteine and lysine residues may be either deleted or substituted with other amino acid residues and first attachment sites may be added to these proteins. Further, pilin proteins may either be expressed in modified form or may be chemically modified after expression. Similarly, intact pili may be harvested from bacteria and then modified chemically.

[0181] In another embodiment, pili or pilus-like structures are harvested from bacteria (*e.g.*, *E. coli*) and used to form vaccine compositions of the invention. One example of pili suitable for preparing vaccine compositions is the Type-1 pilus of *E. coli*, which is formed from pilin monomers having the amino acid sequence set out in SEQ ID NO:146.

[0182] A number of methods for harvesting bacterial pili are known in the art. Bullitt and Makowski (*Biophys. J.* 74:623-632 (1998)), for example, describe a pilus purification method for harvesting P-pili from *E. coli*. According to this method, pili are sheared from hyperpilated *E. coli* containing a P-pilus plasmid and purified by cycles of solubilization and  $\text{MgCl}_2$  (1.0 M) precipitation. A similar purification method is set out below in Example 33.

[0183] Once harvested, pili or pilus-like structures may be modified in a variety of ways. For example, a first attachment site can be added to the pili to which antigens or antigen determinants may be attached through a second attachment site. In other words, bacterial pili or pilus-like structures can be harvested and modified to form non-natural molecular scaffolds.

[0184] Pili or pilus-like structures may also be modified by the attachment of antigens or antigenic determinants in the absence of a non-natural organizer. For example, antigens or antigenic determinants could be linked to naturally occurring

cysteine residues or lysine residues. In such instances, the high order and repetitiveness of a naturally occurring amino acid residue would guide the coupling of the antigens or antigenic determinants to the pili or pilus-like structures. For example, the pili or pilus-like structures could be linked to the second attachment sites of the antigens or antigenic determinants using a heterobifunctional cross-linking agent.

[0185] When structures which are naturally synthesized by organisms (*e.g.*, pili) are used to prepare vaccine compositions of the invention, it will often be advantageous to genetically engineer these organisms so that they produce structures having desirable characteristics. For example, when Type-1 pili of *E. coli* are used, the *E. coli* from which these pili are harvested may be modified so as to produce structures with specific characteristics. Examples of possible modifications of pilin proteins include the insertion of one or more lysine residues, the deletion or substitution of one or more of the naturally resident lysine residues, and the deletion or substitution of one or more naturally resident cysteine residues (*e.g.*, the cysteine residues at positions 44 and 84 in SEQ ID NO:146).

[0186] Further, additional modifications can be made to pilin genes which result in the expression products containing a first attachment site other than a lysine residue (*e.g.*, a *FOS* or *JUN* domain). Of course, suitable first attachment sites will generally be limited to those which do not prevent pilin proteins from forming pili or pilus-like structures suitable for use in vaccine compositions of the invention.

[0187] Pilin genes which naturally reside in bacterial cells can be modified *in vivo* (*e.g.*, by homologous recombination) or pilin genes with particular characteristics can be inserted into these cells. For examples, pilin genes could be introduced into bacterial cells as a component of either a replicable cloning vector or a vector which inserts into the bacterial chromosome. The inserted pilin genes may also be linked to expression regulatory control sequences (*e.g.*, a *lac* operator).

[0188] In most instances, the pili or pilus-like structures used in vaccine compositions of the invention will be composed of single type of a pilin subunit.

Pili or pilus-like structures composed of identical subunits will generally be used because they are expected to form structures which present highly ordered and repetitive antigen arrays.

[0189] However, the compositions of the invention also include vaccines comprising pili or pilus-like structures formed from heterogenous pilin subunits. The pilin subunits which form these pili or pilus-like structures can be expressed from genes naturally resident in the bacterial cell or may be introduced into the cells. When a naturally resident pilin gene and an introduced gene are both expressed in a cell which forms pili or pilus-like structures, the result will generally be structures formed from a mixture of these pilin proteins. Further, when two or more pilin genes are expressed in a bacterial cell, the relative expression of each pilin gene will typically be the factor which determines the ratio of the different pilin subunits in the pili or pilus-like structures.

[0190] When pili or pilus-like structures having a particular composition of mixed pilin subunits is desired, the expression of at least one of the pilin genes can be regulated by a heterologous, inducible promoter. Such promoters, as well as other genetic elements, can be used to regulate the relative amounts of different pilin subunits produced in the bacterial cell and, hence, the composition of the pili or pilus-like structures.

[0191] In addition, while in most instances the antigen or antigenic determinant will be linked to bacterial pili or pilus-like structures by a bond which is not a peptide bond, bacterial cells which produce pili or pilus-like structures used in the compositions of the invention can be genetically engineered to generate pilin proteins which are fused to an antigen or antigenic determinant. Such fusion proteins which form pili or pilus-like structures are suitable for use in vaccine compositions of the invention.

[0192] The inventors surprisingly found that bacterial Pili induced an antibody response dominated by the IgG1 isotype in mice. This type of antibodies is indicative for a Th2 response. Moreover, antigens coupled to Pili also induced a

IgG1 response indicating that coupling of antigens to Pili was sufficient for induction of antigen-specific Th2 responses.

B. Construction of an Antigen or Antigenic Determinant with a Second Attachment Site

[0193] The second element in the compositions of the invention is an antigen or antigenic determinant possessing at least one second attachment site capable of association through at least one non-peptide bond to the first attachment site of the non-natural molecular scaffold. The invention provides for compositions that vary according to the antigen or antigenic determinant selected in consideration of the desired therapeutic effect. Other compositions are provided by varying the molecule selected for the second attachment site.

[0194] However, when bacterial pili, or pilus-like structures, pilin proteins are used to prepare vaccine compositions of the invention, antigens or antigenic determinants may be attached to pilin proteins by the expression of pilin/antigen fusion proteins. Antigen and antigenic determinants may also be attached to bacterial pili, or pilus-like structures, pilin proteins through non-peptide bonds.

[0195] Antigens of the invention may be selected from the group consisting of the following: (a) proteins suited to induce an immune response against cancer cells; (b) proteins suited to induce an immune response against infectious diseases; (c) proteins suited to induce an immune response against allergens, (d) proteins suited to induce an immune response in farm animals, and (e) fragments (*e.g.*, a domain) of any of the proteins set out in (a)-(d).

[0196] In one specific embodiment of the invention, the antigen or antigenic determinant is one that is useful for the prevention of infectious disease. Such treatment will be useful to treat a wide variety of infectious diseases affecting a wide range of hosts, *e.g.*, human, cow, sheep, pig, dog, cat, other mammalian species and non-mammalian species as well. Treatable infectious diseases are well known to those skilled in the art, examples include infections of viral etiology such as HIV, influenza, *Herpes*, viral hepatitis, Epstein Bar, polio, viral encephalitis,

measles, chicken pox, etc.; or infections of bacterial etiology such as pneumonia, tuberculosis, syphilis, etc.; or infections of parasitic etiology such as malaria, trypanosomiasis, leishmaniasis, trichomoniasis, amoebiasis, etc. Thus, antigens or antigenic determinants selected for the compositions of the invention will be well known to those in the medical art; examples of antigens or antigenic determinants include the following: the HIV antigens gp140 and gp160; the influenza antigens hemagglutinin and neuraminidase, Hepatitis B surface antigen, circumsporozoite protein of malaria.

[0197] In another specific embodiment, compositions of the invention are an immunotherapeutic that may be used for the treatment of allergies or cancer.

[0198] The selection of antigens or antigenic determinants for compositions and methods of treatment for allergies would be known to those skilled in the medical art treating such disorders; representative examples of this type of antigen or antigenic determinant include the following: bee venom phospholipase A<sub>2</sub>, Bet v I (birch pollen allergen), 5 Dol m V (white-faced hornet venom allergen), Der p I (House dust mite allergen).

[0199] The selection of antigens or antigenic determinants for compositions and methods of treatment for cancer would be known to those skilled in the medical art treating such disorders; representative examples of this type of antigen or antigenic determinant include the following: Her2 (breast cancer), GD2 (neuroblastoma), EGF-R (malignant glioblastoma), CEA (medullary thyroid cancer), CD52 (leukemia).

[0200] In a particular embodiment of the invention, the antigen or antigenic determinant is selected from the group consisting of: (a) a recombinant protein of HIV, (b) a recombinant protein of Influenza virus, (c) a recombinant protein of Hepatitis B virus, (d) a recombinant protein of *Toxoplasma*, (e) a recombinant protein of *Plasmodium falciparum*, (f) a recombinant protein of *Plasmodium vivax*, (g) a recombinant protein of *Plasmodium ovale*, (h) a recombinant protein of *Plasmodium malariae*, (i) a recombinant protein of breast cancer cells, (j) a recombinant protein of kidney cancer cells, (k) a recombinant protein of prostate



cancer cells, (l) a recombinant protein of skin cancer cells, (m) a recombinant protein of brain cancer cells, (n) a recombinant protein of leukemia cells, (o) a recombinant profiling, (p) a recombinant protein of bee sting allergy, (q) a recombinant proteins of nut allergy, (r) a recombinant proteins of food allergies, (s) recombinant proteins of asthma, (t) a recombinant protein of *Chlamydia*, and (u) a fragment of any of the proteins set out in (a)-(t).

[0201] Once the antigen or antigenic determinant of the composition is chosen, at least one second attachment site may be added to the molecule in preparing to construct the organized and repetitive array associated with the non-natural molecular scaffold of the invention. Knowledge of what will constitute an appropriate second attachment site will be known to those skilled in the art. Representative examples of second attachment sites include, but are not limited to, the following: an antigen, an antibody or antibody fragment, biotin, avidin, streptavidin, a receptor, a receptor ligand, a ligand, a ligand-binding protein, an interacting leucine zipper polypeptide, an amino group, a chemical group reactive to an amino group; a carboxyl group, chemical group reactive to a carboxyl group, a sulfhydryl group, a chemical group reactive to a sulfhydryl group, or a combination thereof.

[0202] The association between the first and second attachment sites will be determined by the characteristics of the respective molecules selected but will comprise at least one non-peptide bond. Depending upon the combination of first and second attachment sites, the nature of the association may be covalent, ionic, hydrophobic, polar, or a combination thereof.

[0203] In one embodiment of the invention, the second attachment site may be the *FOS* leucine zipper protein domain or the *JUN* leucine zipper protein domain.

[0204] In a more specific embodiment of the invention, the second attachment site selected is the *FOS* leucine zipper protein domain, which associates specifically with the *JUN* leucine zipper protein domain of the non-natural molecular scaffold of the invention. The association of the *JUN* and *FOS* leucine zipper protein domains provides a basis for the formation of an organized and repetitive antigen

or antigenic determinant array on the surface of the scaffold. The *FOS* leucine zipper protein domain may be fused in frame to the antigen or antigenic determinant of choice at either the amino terminus, carboxyl terminus or internally located in the protein if desired.

[0205] Several *FOS* fusion constructs are provided for exemplary purposes. Human growth hormone (Example 4), bee venom phospholipase A<sub>2</sub> (PLA) (Example 9), ovalbumin (Example 10) and HIV gp140 (Example 12).

[0206] In order to simplify the generation of *FOS* fusion constructs, several vectors are disclosed that provide options for antigen or antigenic determinant design and construction (*see* Example 6). The vectors pAV1-4 were designed for the expression of *FOS* fusion in *E. coli*; the vectors pAV5 and pAV6 were designed for the expression of *FOS* fusion proteins in eukaryotic cells. Properties of these vectors are briefly described:

[0207] 1. pAV1: This vector was designed for the secretion of fusion proteins with *FOS* at the C-terminus into the *E. coli* periplasmic space. The gene of interest (g.o.i.) may be ligated into the StuI/NotI sites of the vector.

[0208] 2. pAV2: This vector was designed for the secretion of fusion proteins with *FOS* at the N-terminus into the *E. coli* periplasmic space. The gene of interest (g.o.i.) ligated into the NotI/EcoRV (or NotI/HindIII) sites of the vector.

[0209] 3. pAV3: This vector was designed for the cytoplasmic production of fusion proteins with *FOS* at the C-terminus in *E. coli*. The gene of interest (g.o.i.) may be ligated into the EcoRV/NotI sites of the vector.

[0210] 4. pAV4: This vector is designed for the cytoplasmic production of fusion proteins with *FOS* at the N-terminus in *E. coli*. The gene of interest (g.o.i.) may be ligated into the NotI/EcoRV (or NotI/HindIII) sites of the vector. The N-terminal methionine residue is proteolytically removed upon protein synthesis (Hirel *et al.*, *Proc. Natl. Acad. Sci. USA* 86:8247-8251 (1989)).

[0211] 5. pAV5: This vector was designed for the eukaryotic production of fusion proteins with *FOS* at the C-terminus. The gene of interest (g.o.i.) may be inserted between the sequences coding for the hGH signal sequence and the *FOS*

domain by ligation into the Eco47III/NotI sites of the vector. Alternatively, a gene containing its own signal sequence may be fused to the *FOS* coding region by ligation into the StuI/NotI sites.

[0212] 6. pAV6: This vector was designed for the eukaryotic production of fusion proteins with *FOS* at the N-terminus. The gene of interest (g.o.i.) may be ligated into the NotI/StuI (or NotI/HindIII) sites of the vector.

[0213] As will be understood by those skilled in the art, the construction of a *FOS*-antigen or -antigenic determinant fusion protein may include the addition of certain genetic elements to facilitate production of the recombinant protein. Example 4 provides guidance for the addition of certain *E. coli* regulatory elements for translation, and Example 7 provides guidance for the addition of a eukaryotic signal sequence. Other genetic elements may be selected, depending on the specific needs of the practitioner.

[0214] The invention is also seen to include the production of the *FOS*-antigen or *FOS*-antigenic determinant fusion protein either in bacterial (Example 5) or eukaryotic cells (Example 8). The choice of which cell type in which to express the fusion protein is within the knowledge of the skilled artisan, depending on factors such as whether post-translational modifications are an important consideration in the design of the composition.

[0215] As noted previously, the invention discloses various methods for the construction of a *FOS*-antigen or *FOS*-antigenic determinant fusion protein through the use of the pAV vectors. In addition to enabling prokaryotic and eukaryotic expression, these vectors allow the practitioner to choose between N- and C-terminal addition to the antigen of the *FOS* leucine zipper protein domain. Specific examples are provided wherein N- and C-terminal *FOS* fusions are made to PLA (Example 9) and ovalbumin (Example 10). Example 11 demonstrates the purification of the PLA and ovalbumin *FOS* fusion proteins.

[0216] In a more specific embodiment, the invention is drawn to an antigen or antigenic determinant encoded by the HIV genome. More specifically, the HIV antigen is gp140. As provided for in Examples 11-15, HIV gp140 may be created

with a FOS leucine zipper protein domain and the fusion protein synthesized and purified for attachment to the non-natural molecular scaffold of the invention. As one skilled in the art would know, other HIV antigens or antigenic determinants may be used in the creation of a composition of the invention.

[0217] In a more specific embodiment of the invention, the second attachment site selected is a cysteine residue, which associates specifically with a lysine residue of the non-natural molecular scaffold of the invention. The chemical linkage of the lysine residue (Lys) and cysteine residue (Cys) provides a basis for the formation of an organized and repetitive antigen or antigenic determinant array on the surface of the scaffold. The cysteine residue may be engineered in frame to the antigen or antigenic determinant of choice at either the amino terminus, carboxyl terminus or internally located in the protein if desired. By way of example, PLA and HIV gp140 are provided with a cysteine residue for linkage to a lysine residue first attachment site.

#### C. Preparation of the AlphaVaccine Particles

[0218] The invention provides novel compositions and methods for the construction of ordered and repetitive antigen arrays. As one of skill in the art would know, the conditions for the assembly of the ordered and repetitive antigen array depend to a large extent on the specific choice of the first attachment site of the non-natural molecular scaffold and the specific choice of the second attachment site of the antigen or antigenic determinant. Thus, practitioner choice in the design of the composition (*i.e.*, selection of the first and second attachment sites, antigen and non-natural molecular scaffold) will determine the specific conditions for the assembly of the AlphaVaccine particle (the ordered and repetitive antigen array and non-natural molecular scaffold combined). Information relating to assembly of the AlphaVaccine particle is well within the working knowledge of the practitioner, and numerous references exist to aid the practitioner (*e.g.*, Sambrook, J. *et al.*, eds., MOLECULAR CLONING, A

LABORATORY MANUAL, 2nd. edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); Ausubel, F. *et al.*, eds., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John H. Wiley & Sons, Inc. (1997); Celis, J., ed., CELL BIOLOGY, Academic Press, 2<sup>nd</sup> edition, (1998); Harlow, E. and Lane, D., "Antibodies: A Laboratory Manual," Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1988), all of which are incorporated herein by reference.

[0219] In a specific embodiment of the invention, the *JUN* and *FOS* leucine zipper protein domains are utilized for the first and second attachment sites of the invention, respectively. In the preparation of AlphaVaccine particles, antigen must be produced and purified under conditions to promote assembly of the ordered and repetitive antigen array onto the non-natural molecular scaffold. In the particular *JUN/FOS* leucine zipper protein domain embodiment, the *FOS*-antigen or *FOS*-antigenic determinant should be treated with a reducing agent (*e.g.*, Dithiothreitol (DTT)) to reduce or eliminate the incidence of disulfide bond formation (Example 15).

[0220] For the preparation of the non-natural molecular scaffold (*i.e.*, recombinant Sinbis virus) of the *JUN/FOS* leucine zipper protein domain embodiment, recombinant E2-*JUN* viral particles should be concentrated, neutralized and treated with reducing agent (*see* Example 16).

[0221] Assembly of the ordered and repetitive antigen array in the *JUN/FOS* embodiment is done in the presence of a redox shuffle. E2-*JUN* viral particles are combined with a 240 fold molar excess of *FOS*-antigen or *FOS*-antigenic determinant for 10 hours at 4°C. Subsequently, the AlphaVaccine particle is concentrated and purified by chromatography (Example 16).

[0222] In another embodiment of the invention, the coupling of the non-natural molecular scaffold to the antigen or antigenic determinant may be accomplished by chemical cross-linking. In a specific embodiment, the chemical agent is a heterobifunctional cross-linking agent such as  $\epsilon$ -maleimidocaproic acid N-hydroxysuccinimide ester (Tanimori *et al.*, *J. Pharm. Dyn.* 4:812 (1981); Fujiwara *et al.*, *J. Immunol. Meth.* 45:195 (1981)), which contains (1) a succinimide group

reactive with amino groups and (2) a maleimide group reactive with SH groups. A heterologous protein or polypeptide of the first attachment site may be engineered to contain one or more lysine residues that will serve as a reactive moiety for the succinimide portion of the heterobifunctional cross-linking agent. Once chemically coupled to the lysine residues of the heterologous protein, the maleimide group of the heterobifunctional cross-linking agent will be available to react with the SH group of a cysteine residue on the antigen or antigenic determinant. Antigen or antigenic determinant preparation in this instance may require the engineering of a cysteine residue into the protein or polypeptide chosen as the second attachment site so that it may be reacted to the free maleimide function on the cross-linking agent bound to the non-natural molecular scaffold first attachment sites. Thus, in such an instance, the heterobifunctional cross-linking agent binds to a first attachment site of the non-natural molecular scaffold and connects the scaffold to a second binding site of the antigen or antigenic determinant.

### 3. Compositions, Vaccines, and the Administration Thereof, and Methods of Treatment

[0223] In one embodiment, the invention provides vaccines for the prevention of infectious diseases in a wide range of species, particularly mammalian species such as human, monkey, cow, dog, cat, horse, pig, etc. Vaccines may be designed to treat infections of viral etiology such as HIV, influenza, *Herpes*, viral hepatitis, Epstein Bar, polio, viral encephalitis, measles, chicken pox, etc.; or infections of bacterial etiology such as pneumonia, tuberculosis, syphilis, etc.; or infections of parasitic etiology such as malaria, trypanosomiasis, leishmaniasis, trichomoniasis, amoebiasis, etc.

[0224] In another embodiment, the invention provides vaccines for the prevention of cancer in a wide range of species, particularly mammalian species such as human, monkey, cow, dog, cat, horse, pig, etc. Vaccines may be designed to treat all types of cancer: lymphomas, carcinomas, sarcomas, melanomas, etc.

[0225] In another embodiment of the invention, compositions of the invention may be used in the design of vaccines for the treatment of allergies. Antibodies of the IgE isotype are important components in allergic reactions. Mast cells bind IgE antibodies on their surface and release histamines and other mediators of allergic response upon binding of specific antigen to the IgE molecules bound on the mast cell surface. Inhibiting production of IgE antibodies, therefore, is a promising target to protect against allergies. This should be possible by attaining a desired T helper cell response. T helper cell responses can be divided into type 1 ( $T_H1$ ) and type 2 ( $T_H2$ ) T helper cell responses (Romagnani, *Immunol. Today* 18:263-266 (1997)).  $T_H1$  cells secrete interferon-gamma and other cytokines which trigger B cells to produce protective IgG antibodies. In contrast, a critical cytokine produced by  $T_H2$  cells is IL-4, which drive B cells to produce IgE. In many experimental systems, the development of  $T_H1$  and  $T_H2$  responses is mutually exclusive since  $T_H1$  cells suppress the induction of  $T_H2$  cells and *vice versa*. Thus, antigens that trigger a strong  $T_H1$  response simultaneously suppress the development of  $T_H2$  responses and hence the production of IgE antibodies. Interestingly, virtually all viruses induce a  $T_H1$  response in the host and fail to trigger the production of IgE antibodies (Coutelier *et al.*, *J. Exp. Med.* 165:64-69 (1987)). This isotype pattern is not restricted to live viruses but has also been observed for inactivated or recombinant viral particles (Lo-Man *et al.*, *Eur. J. Immunol.* 28:1401-1407 (1998)). Thus, by using the processes of the invention (*e.g.*, AlphaVaccine Technology), viral particles can be decorated with various allergens and used for immunization. Due to the resulting "viral structure" of the allergen, a  $T_H1$  response will be elicited, "protective" IgG antibodies will be produced, and the production of IgE antibodies which cause allergic reactions will be prevented. Since the allergen is presented by viral particles which are recognized by a different set of helper T cells than the allergen itself, it is likely that the allergen-specific IgG antibodies will be induced even in allergic individuals harboring pre-existing  $T_H2$  cells specific for the allergen. The presence of high concentrations of IgG antibodies may prevent binding of allergens to mast cell

bound IgE, thereby inhibiting the release of histamine. Thus, presence of IgG antibodies may protect from IgE mediated allergic reactions. Typical substances causing allergies include: grass, ragweed, birch or mountain cedar pollens, house dust, mites, animal danders, mold, insect venom or drugs (*e.g.*, penicillin). Thus, immunization of individuals with allergen-decorated viral particles should be beneficial not only before but also after the onset of allergies. Food allergies are also very common, and immunization of subjects with particles decorated with food allergens should be useful for the treatment of these allergies.

[0226] In another embodiment, the invention relates to the induction of specific Th type 2 (Th2) cells. The inventors surprisingly found that bacterial Pili induce an antibody response dominated by the IgG1 isotype in mice, indicative of a Th2 response. Antigens coupled to Pili also induced a IgG1 response indicating that coupling of antigens to Pili was sufficient for induction of antigen-specific Th2 response. Many chronic diseases in humans and animals, such as arthritis, colitis, diabetes and multiple sclerosis are dominated by Th1 response, where T cells secrete IFN $\gamma$  and other pro-inflammatory cytokines precipitating disease. By contrast, Th2 cells secrete IL-4, IL-13 and also IL-10. The latter cytokine is usually associated with immunosuppression and there is good evidence that specific Th2 cells can suppress chronic diseases, such as arthritis, colitis, diabetes and multiple sclerosis *in vivo*. Thus, induction of antigen-specific Th2 cells is desirable for the treatment of such chronic diseases.

[0227] It is known that induction of therapeutic self-specific antibodies may allow treating a variety of diseases. It is, *e.g.*, known that anti-TNF antibodies can ameliorate symptoms in arthritis or colitis and antibodies specific for the A $\beta$ -peptide may remove plaques from the brain of Alzheimers patients. It will usually be beneficial for the patient if such antibodies can be induced in the absence of a pro-inflammatory Th1 response. Thus, self antigens coupled to Pili that induce a strong antibody response but no Th1 response may be optimal for such immunotherapy.



- [0228] In a preferred embodiment, the antigen is the amyloid beta peptide ( $A\beta_{1-42}$ ) (DAEFRHDSGYEVHHQKL VFFAEDVGSNKGAIIGLMVGGVVIA (SEQ ID NO: 174), or a fragment thereof. The amyloid beta protein is SEQ ID NO: 172. The amyloid beta precursor protein is SEQ ID NO: 173.
- [0229] The amyloid B peptide ( $A\beta_{1-42}$ ) has a central role in the neuropathology of Alzheimers disease. Region specific, extracellular accumulation of  $A\beta$  peptide is accompanied by microgliosis, cytoskeletal changes, dystrophic neuritis and synaptic loss. These pathological alterations are thought to be linked to the cognitive decline that defines the disease.
- [0230] In a mouse model of Alzheimer disease, transgenic animals engineered to produce  $A\beta_{1-42}$  (PDAPP-mice), develop plaques and neuron damage in their brains. Recent work has shown immunization of young PDAPP-mice, using  $A\beta_{1-42}$ , resulted in inhibition of plaque formation and associated dystrophic neuritis (Schenk, D. *et al.*, *Nature* 400:173-77 (1999)).
- [0231] Furthermore immunization of older PDAPP mice that had already developed AD-like neuropathologies, reduced the extent and progression of the neuropathologies. The immunization protocol for these studies was as follows; peptide was dissolved in aqueous buffer and mixed 1:1 with complete Freund's adjuvant (for primary dose) to give a peptide concentration of 100 $\mu$ g/dose. Subsequent boosts used incomplete Freund's adjuvant. Mice received 11 immunizations over an 11 month period. Antibodies titres greater than 1:10 000 were achieved and maintained. Hence, immunization may be an effective prophylactic and therapeutic action against Alzheimer disease.
- [0232] In another study, peripherally administered antibodies raised against  $A\beta_{1-42}$ , were able to cross the blood-brain barrier, bind  $A\beta$  peptide, and induce clearance of pre-existing amyloid (Bard, F. *et al.*, *Nature Medicine* 6: 916-19 (2000)). This study utilized either polyclonal antibodies raised against  $A\beta_{1-42}$ , or monoclonal antibodies raised against synthetic fragments derived from different regions of  $A\beta$ . Thus induction of antibodies can be considered as a potential therapeutic treatment for Alzheimer disease.

[0233] In another more specific embodiment, the invention is drawn to vaccine compositions comprising at least one antigen or antigenic determinant encoded by an Influenza viral nucleic acid, and the use of such vaccine compositions to elicit immune responses. In an even more specific embodiment, the Influenza antigen or antigenic determinant may be an M2 protein (*e.g.*, an M2 protein having the amino acids shown in SEQ ID NO: 171, GenBank Accession No. P06821, or in SEQ ID NO: 170, PIR Accession No. MFIV62, or fragment thereof (*e.g.*, amino acids from about 2 to about 24 in SEQ ID NO: 171, the amino acid sequence in SEQ ID NO: 170. Further, influenza antigens or antigenic determinants may be coupled to pili or pilus-like structures. Portions of an M2 protein (*e.g.*, an M2 protein having the amino acid sequence in SEQ ID NO: 170), as well as other proteins against which an immunological response is sought, suitable for use with the invention may comprise, or alternatively consist of, peptides of any number of amino acids in length but will generally be at least 6 amino acids in length (*e.g.*, peptides 6, 7, 8, 9, 10, 12, 15, 18, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 97 amino acids in length).

[0234] In an even more specific embodiment, the Influenza antigen or antigenic determinant may be an M2 protein (*e.g.*, an M2 protein having the amino acids shown in SEQ ID NO: 170, GenBank Accession No. P06821, or in SEQ ID NO: 212, PIR Accession No. MFIV62, or fragment thereof (*e.g.*, amino acids from about 2 to about 24 in SEQ ID NO: 171, the amino acid sequence in SEQ ID NO: 170).

[0235] As would be understood by one of ordinary skill in the art, when compositions of the invention are administered to an individual, they may be in a composition which contains salts, buffers, adjuvants, or other substances which are desirable for improving the efficacy of the composition. Examples of materials suitable for use in preparing pharmaceutical compositions are provided in numerous sources including REMINGTON'S PHARMACEUTICAL SCIENCES (Osol, A, ed., Mack Publishing Co., (1980)).

[0236] Compositions of the invention are said to be "pharmacologically acceptable" if their administration can be tolerated by a recipient individual. Further, the compositions of the invention will be administered in a "therapeutically effective amount" (*i.e.*, an amount that produces a desired physiological effect).

[0237] The compositions of the present invention may be administered by various methods known in the art, but will normally be administered by injection, infusion, inhalation, oral administration, or other suitable physical methods. The compositions may alternatively be administered intramuscularly, intravenously, or subcutaneously. Components of compositions for administration include sterile aqueous (*e.g.*, physiological saline) or non-aqueous solutions and suspensions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Carriers or occlusive dressings can be used to increase skin permeability and enhance antigen absorption.

[0238] The present invention also provides a composition comprising a bacterial pilin polypeptide to which an antigen or antigenic determinant has been attached by a covalent bond.

[0239] The present invention also provides a composition comprising a fragment of a bacteriophage coat protein to which an antigen or antigenic determinant has been attached by a covalent bond.

[0240] The present invention also provides a composition comprising (a) non-natural molecular scaffold comprising (i) a core particle selected from the group consisting of (1) a bacterial pilus or pilin protein; and (2) a recombinant form of a bacterial pilus or pilin protein; and (ii) an organizer comprising at least one first attachment site, wherein the organizer is connected to the core particle by at least one covalent bond; and (b) an antigen or antigenic determinant with at least one second attachment site, the second attachment site being selected from the group consisting of (i) an attachment site not naturally occurring with the antigen or antigenic determinant; and (ii) an attachment site naturally occurring with the antigen or antigenic determinant, wherein the second attachment site is capable of

association through at least one non-peptide bond to the first attachment site; and wherein the antigen or antigenic determinant and the scaffold interact through the association to form an ordered and repetitive antigen array.

[0241] The present invention also provides a composition comprising (a) a non-natural molecular scaffold comprising (i) a core particle selected from the group consisting of: (1) a bacterial pilus; and (2) a recombinant form of a bacterial pilus; and (ii) an organizer comprising at least one first attachment site, wherein the organizer is connected to the core particle by at least one covalent bond; and (b) an antigen or antigenic determinant with at least one second attachment site, the second attachment site being selected from the group consisting of (i) an attachment site not naturally occurring with the antigen or antigenic determinant; and (ii) an attachment site naturally occurring with the antigen or antigenic determinant, wherein the second attachment site is capable of association through at least one non-peptide bond to the first attachment site; and wherein the antigen or antigenic determinant and the scaffold interact through the association to form an ordered and repetitive antigen array.

[0242] The present invention also provides a composition comprising (a) a non-natural molecular scaffold comprising (i) a virus-like particle that is a dimer or a multimer of a polypeptide comprising amino acids 1-147 of SEQ ID NO:158 as core particle; and (ii) an organizer comprising at least one first attachment site, wherein the organizer is connected to the core particle by at least one covalent bond; and (b) an antigen or antigenic determinant with at least one second attachment site, the second attachment site being selected from the group consisting of (i) an attachment site not naturally occurring with the antigen or antigenic determinant; and (ii) an attachment site naturally occurring with the antigen or antigenic determinant, wherein the second attachment site is capable of association through at least one non-peptide bond to the first attachment site; and wherein the antigen or antigenic determinant and the scaffold interact through the association to form an ordered and repetitive antigen array.

[0243] The present invention also provides a pharmaceutical composition comprising any of compositions of the present invention, and a pharmaceutically acceptable carrier.

[0244] The present invention also provides a vaccine composition comprising any of compositions of the present invention. The vaccine composition may further comprise at least one adjuvant. The present invention also provides a method of immunizing, comprising administering to a subject a vaccine composition of the present invention.

[0245] The present invention also provides a composition comprising (a) a non-natural molecular scaffold comprising (i) Hepatitis B virus capsid protein comprising an amino acid sequence selected from the group consisting of (1) the amino acid sequence of SEQ ID NO:89, (2) the amino acid sequence of SEQ ID NO:90 (3) the amino acid sequence of SEQ ID NO:93, (4) the amino acid sequence of SEQ ID NO:98, (5) the amino acid sequence of SEQ ID NO:99, (6) the amino acid sequence of SEQ ID NO:102, (7) the amino acid sequence of SEQ ID NO:104, (8) the amino acid sequence of SEQ ID NO:105, (9) the amino acid sequence of SEQ ID NO:106, (10) the amino acid sequence of SEQ ID NO:119, (11) the amino acid sequence of SEQ ID NO:120, (12) the amino acid sequence of SEQ ID NO:123, (13) the amino acid sequence of SEQ ID NO:125, (14) the amino acid sequence of SEQ ID NO:131, (15) the amino acid sequence of SEQ ID NO:132, (16) the amino acid sequence of SEQ ID NO:134, (17) the amino acid sequence of SEQ ID NO:157, and (18) the amino acid sequence of SEQ ID NO:158; and (ii) an organizer comprising at least one first attachment site, wherein the organizer is connected to the core particle by at least one covalent bond; and (b) an antigen or antigenic determinant with at least one second attachment site, the second attachment site being selected from the group consisting of (i) an attachment site not naturally occurring with the antigen or antigenic determinant; and (ii) an attachment site naturally occurring with the antigen or antigenic determinant, wherein the second attachment site is capable of association through at least one non-peptide bond to the first attachment site; and

wherein the antigen or antigenic determinant and the scaffold interact through the association to form an ordered and repetitive antigen array. Preferably, the organizer is a polypeptide or residue thereof, wherein the second attachment site is a polypeptide or residue thereof, and wherein the first attachment site is a lysine residue and the second attachment site is a cysteine residue. Preferably, one or more cysteine residues of the Hepatitis B virus capsid protein have been either deleted or substituted with another amino acid residue. Preferably, the cysteine residues corresponding to amino acids 48 and 107 in SEQ ID NO:134 have been either deleted or substituted with another amino acid residue.

[0246] The present invention also provides a composition comprising: (1) a non-natural molecular scaffold comprising (i) a core particle selected from the group consisting of (1) a bacterial pilus, and (2) a recombinant form of a bacterial pilus or pilin protein; and (ii) an organizer comprising at least one first attachment site, wherein the organizer is connected to the core particle by at least one covalent bond; and (2) an antigen or antigenic determinant with at least one second attachment site, the second attachment site being selected from the group consisting of (i) an attachment site not naturally occurring with the antigen or antigenic determinant, and (ii) an attachment site naturally occurring with the antigen or antigenic determinant, wherein the second attachment site is capable of association through at least one non-peptide bond to the first attachment site, wherein the antigen or antigenic determinant and the scaffold interact through the association to form an ordered and repetitive antigen array, and wherein the antigen or antigenic determinant is selected from the group consisting of an influenza M2 peptide, the GRA2 polypeptide, the DP178c peptide, the tumor necrosis factor polypeptide, a tumor necrosis factor peptide, the B2 peptide, the D2 peptide, and the A $\beta$  peptide.

[0247] In the compositions and vaccines of the present invention, for a covalent bond between a first and second attachment site, the covalent bond is preferably not a peptide bond.

[0248] If a bacterial pilus is present in a composition or vaccine of the present invention, the pilus is preferably a Type-1 pilus of *Eschericia coli*. More preferably, pilin subunits of the Type-1 pilus comprises the amino acid sequence shown in SEQ ID NO:146. Preferably, the bacterial pilus and the antigen or antigen determinant are attached via either a naturally or non-naturally occurring attachment. Preferably, the first attachment site will be a lysine residue, while the second attachment site will be a cysteine residue present or engineered on the antigen. If the attachment comprises interacting leucine zipper polypeptides, the polypeptides are preferably JUN and/or FOS leucine zipper polypeptides.

[0249] In the compositions and vaccines of the present invention that comprise an organizer having a first attachment site, attached to the second attachment site on the antigen, the organizer is preferably a polypeptide or a residue thereof, and the second attachment site is preferably a polypeptide or a residue thereof. More preferably, the first and/or the second attachment sites comprise an antigen and an antibody or antibody fragment thereto, biotin and avidin, streptavidin and biotin, a receptor and its ligand, a ligand-binding protein and its ligand, interacting leucine zipper polypeptides, an amino group and a chemical group reactive thereto, a carboxyl group and a chemical group reactive thereto, a sulfhydryl group and a chemical group reactive thereto, or a combination thereof. More preferably, the first attachment site is an amino group, and the second attachment site is a sulfhydryl group.

[0250] In the compositions and vaccines of the present invention, the antigen is preferably selected from the group consisting of a protein suited to induce an immune response against cancer cells, a protein suited to induce an immune response against infectious diseases, a protein suited to induce an immune response against allergens, and a protein suited to induce an immune response in farm animals. Preferably, the antigen induces an immune response against one or more allergens. More preferably, the antigen is a recombinant protein of HIV, a recombinant protein of Influenza virus, a recombinant protein of Hepatitis C virus, a recombinant protein of Toxoplasma, a recombinant protein of Plasmodium.

falciparum, a recombinant protein of Plasmodium vivax, a recombinant protein of Plasmodium ovale, a recombinant protein of Plasmodium malariae, a recombinant protein of breast cancer cells, a recombinant protein of kidney cancer cells, a recombinant protein of prostate cancer cells, a recombinant protein of skin cancer cells, a recombinant protein of brain cancer cells, a recombinant protein of leukemia cells, a recombinant protein of bee sting allergy, a recombinant protein of nut allergy, a recombinant protein of food allergies, or a recombinant protein of asthma, or a recombinant protein of Chlamydia.

[0251] In the method of immunizing provided by the present invention, the immunization produces an immune response in the subject. Preferably, the immunization produces a humoral immune response, a cellular immune response, a humoral and a cellular immune response, or a protective immune response.

[0252] In the compositions and vaccines of the present invention, the antigen or antigenic determinant is attached to the non-natural molecular scaffold through the first attachment site, to form an antigen array or antigenic determinant array. Preferably, the array is ordered and/or repetitive.

[0253] In the compositions and vaccines of the present invention, the first and/or the second attachment sites are preferably attached via either a non-naturally occurring attachment, or by an attachment comprising interacting leucine zipper polypeptides. More preferably, the interacting leucine zipper polypeptides are JUN and/or FOS leucine zipper polypeptides.

[0254] The present invention also provides a method for making the compositions and vaccines of the present invention, comprising combining the antigen or antigenic determinant with the non-natural molecular scaffold through the first attachment site and organizer present on the non-natural molecular scaffold.

[0255] In addition to vaccine technologies, other embodiments of the invention are drawn to methods of medical treatment for cancer, allergies, and chronic diseases.

[0256] Following is a protocol for analyzing pili by SDS-PAGE Analysis. Add trichloroacetic acid to a final concentration of 10% to the pili solution containing



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approx. 50 ug of pili. Vortex and incubate for 10 minutes on ice. Centrifuge at maximal speed for 5 minutes in a microcentrifuge. Discard the supernatant and resuspend the pellet in 50 ul of a 8.5 M guanidiniumhydrochloride, pH 3 solution. Heat the sample for 15 minutes at 70°C. Precipitate the protein by adding 1.5 ml of Ethanol precooled at -20°C, and centrifuge 5 minutes at RT at maximal speed. Resuspend the pellet in 15 ul of a 10 mM Tris, pH 8 buffer. Add SDS-PAGE sample buffer, vortex shortly and heat the sample 10 minutes at 100°C. Load the sample on a 12% gel.

#### EXAMPLES

[0257] Enzymes and reagents used in the experiments that follow included: T4 DNA ligase obtained from New England Biolabs; Taq DNA Polymerase, QIAprep Spin Plasmid Kit, QIAGEN Plasmid Midi Kit, QiaExII Gel Extraction Kit, QIAquick PCR Purification Kit obtained from QIAGEN; QuickPrep Micro mRNA Purification Kit obtained from Pharmacia; SuperScript One-step RT PCR Kit, fetal calf serum (FCS), bacto-tryptone and yeast extract obtained from Gibco BRL; Oligonucleotides obtained from Microsynth (Switzerland); restriction endonucleases obtained from Boehringer Mannheim, New England Biolabs or MBI Fermentas; Pwo polymerase and dNTPs obtained from Boehringer Mannheim. HP-1 medium was obtained from Cell culture technologies (Glattbrugg, Switzerland). All standard chemicals were obtained from Fluka-Sigma-Aldrich, and all cell culture materials were obtained from TPP.

[0258] DNA manipulations were carried out using standard techniques. DNA was prepared according to manufacturer instruction either from a 2 ml bacterial culture using the QIAprep Spin Plasmid Kit or from a 50 ml culture using the QIAGEN Plasmid Midi Kit. For restriction enzyme digestion, DNA was incubated at least 2 hours with the appropriate restriction enzyme at a concentration of 5-10 units (U) enzyme per mg DNA under manufacturer recommended conditions (buffer and temperature). Digests with more than one enzyme were performed simultaneously if reaction conditions were appropriate for

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all enzymes, otherwise consecutively. DNA fragments isolated for further manipulations were separated by electrophoresis in a 0.7 to 1.5% agarose gel, excised from the gel and purified with the QiaExII Gel Extraction Kit according to the instructions provided by the manufacturer. For ligation of DNA fragments, 100 to 200 pg of purified vector DNA were incubated overnight with a threefold molar excess of the insert fragment at 16°C in the presence of 1 U T4 DNA ligase in the buffer provided by the manufacturer (total volume: 10-20 µl). An aliquot (0.1 to 0.5 µl) of the ligation reaction was used for transformation of *E. coli* XL1-Blue (Stratagene). Transformation was done by electroporation using a Gene Pulser (BioRAD) and 0.1 cm Gene Pulser Cuvettes (BioRAD) at 200 Ω, 25 µF, 1.7 kV. After electroporation, the cells were incubated with shaking for 1 h in 1 ml S.O.B. medium (Miller, 1972) before plating on selective S.O.B. agar.

#### EXAMPLE 1:

##### Insertion of the JUN amphipathic helix domain within E2

[0259] In the vector pTE5'2J (Hahn *et al.*, *Proc. Natl. Acad. Sci. USA* 89:2679-2683, (1992)), *Mlu*I and a *Bst*EII restriction enzyme sites were introduced between codons 71 (Gln) and 74 (Thr) of the structural protein E2 coding sequence, resulting in vector pTE5'2JBM. Introduction of these restriction enzymes sites was done by PCR using the following oligonucleotides:

Oligo 1:

E2insBstEII/BssHII:

5'-ggggACGCGTGCAGCaggtaaccaccgTTAAAGAAGGCACC-3' (SEQ ID NO:1)

Oligo 2:

E2insMluIStuI:

5'-cgggtggttaccTGCTGCACGCGTTGCTTAAGCGACATGTAGCGG-3' (SEQ ID NO:2)

Oligo 3:

E2insStuI: 5'-CCATGAGGCCTACGATACCC-3' (SEQ ID NO:3)

Oligo4:

E2insBssHII: 5'-GGCACTCACGGCGCGCTTTACAGGC-3' (SEQ ID NO:4)

- [0260] For the PCR reaction, 100 pmol of each oligo was used with 5 ng of the template DNA in a 100 µl reaction mixture containing 4 units of Taq or Pwo polymerase, 0.1 mM dNTPs and 1.5 mM MgSO<sub>4</sub>. All DNA concentrations were determined photometrically using the GeneQuant apparatus (Pharmacia). Polymerase was added directly before starting the PCR reaction (starting point was 95°C). Temperature cycling was done in the following manner and order: 95°C for 2 minutes; 5 cycles of 95°C (45 seconds), 53°C (60 seconds), 72°C (80 seconds); and 25 cycles of 95°C (45 seconds), 57°C (60 seconds), 72°C (80 seconds).
- [0261] The two PCR fragments were analyzed and purified by agarose gelelectrophoresis. Assembly PCR of the two PCR fragments using oligo 3 and 4 for amplification was carried out to obtain the final construct.
- [0262] For the assembly PCR reaction, 100 pmol of each oligo was used with 2 ng of the purified PCR fragments in a 100 µl reaction mixture containing 4 units of Taq or Pwo polymerase, 0.1 mM dNTPs and 1.5 mM MgSO<sub>4</sub>. All DNA concentrations were determined photometrically using the GeneQuant apparatus (Pharmacia). Polymerase was added directly before starting the PCR reaction (starting point was 95°C). Temperature cycling was done in the following manner and order: 95°C for 2 minutes; 5 cycles of 95°C (45 seconds), 57°C (60 seconds), 72°C (90 seconds); and 25 cycles of 95°C (45 seconds), 59°C (60 seconds), 72°C (90 seconds).
- [0263] The final PCR product was purified using Qia spin PCR columns (Qiagen) and digested in an appropriate buffer using 10 units each of BssHII and StuI restriction endonucleases for 12 hours at 37°C. The DNA fragments were gel-purified and ligated into BssHII/StuI digested and gel-purified pTE5'2J vector (Hahn *et al.*, *Proc. Natl. Acad. Sci. USA* 89:2679-2683). The correct insertion of the PCR product was first analyzed by BstEII and MluI restriction analysis and then by DNA sequencing of the PCR fragment.

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[0264] The DNA sequence coding for the *JUN* amphipathic helix domain was PCR-amplified from vector pJuFo (Cramer and Suter, *Gene* 137:69 (1993)) using the following oligonucleotides:

Oligo 5:

*JUN*BstEII:

5'-CCTTCTTTAAcgggtggttaccTGCTGGCAACCAACGTGGTTCATGAC-3'  
(SEQ ID NO:5)

Oligo 6:

*MluI**JUN*: 5'-AAGCATGCTGCacgcgtgTGCGGTGGTCGGATCGCCCGGC-3'  
(SEQ ID NO:6)

[0265] For the PCR reaction, 100 pmol of each oligo was used with 5 ng of the template DNA in a 100 µl reaction mixture containing 4 units of Taq or Pwo polymerase, 0.1 mM dNTPs and 1.5 mM MgSO<sub>4</sub>. All DNA concentrations were determined photometrically using the GeneQuant apparatus (Pharmacia). Polymerase was added directly before starting the PCR reaction (starting point was 95°C). Temperature cycling was done in the following order and manner: 95°C for 2 minutes; 5 cycles of 95°C (45 seconds), 60°C (30 seconds), 72°C (25 seconds); and 25 cycles of 95°C (45 seconds), 68°C (30 seconds), 72°C (20 seconds).

[0266] The final PCR product was gel-purified and ligated into EcoRV digested and gel-purified pBluescript II(KS<sup>-</sup>). From the resulting vector, the *JUN* sequence was isolated by cleavage with *MluI*/*Bst*EII purified with QiaExII and ligated into vector pTE5'2JBM (previously cut with the same restriction enzymes) to obtain the vector pTE5'2J:E2*JUN*.

## EXAMPLE 2:

Production of viral particles containing E2-JUN using the pCYTts system

[0267] The structural proteins were PCR amplified using pTE5'2J:E2*JUN* as template and the oligonucleotides XbaIStruct (ctatcaTCTAGAATGAATAGAGGATTCTTTAAC (SEQ ID NO:12)) and StructBsp1201 (tcgaatGGGCCCTCATCTTCGTGTGCTAGTCAG (SEQ ID

NO:87)). For the PCR 100 pmol of each loloigo was used and 5 ng of the template DNA was used in the 100 µl reaction mixture, containing 4 units of Tac or Pwo polymerase, 0.1 mM dNTPs and 1.5 mM MgSO<sub>4</sub>. All DNA concentrations were determined photometrically using the GeneQuant apparatus (Pharmacia). The polymerase was added directly before starting the PCR reaction (starting point was 95°C). The temperature cycles were as follows: 95°C for 3 minutes, followed by 5 cycles of 92°C (30 seconds), 54°C (35 seconds), 72°C (270 seconds) and followed by 25 cycles of 92°C (30 seconds), 63°C (35 seconds), 72°C (270 seconds). The PCR product was gel purified and digested with the restriction enzymes XbaI/Bsp1201 and ligated into vector pCYTts previously cleaved with the same enzymes (WO 99/50432)

[0268] Twenty µg of pCYTtsE2:*JUN* were incubated with 30 U of ScaI in an appropriate buffer for at least 4 hours at 37°C. The reaction was stopped by phenol/chloroform extraction, followed by an isopropanol precipitation of the linerized DNA. The restriction reaction was checked by agarose gel eletrophoresis. For the transfection, 5.4 µg of linearized pCYTtsE2:*JUN* was mixed with 0.6 µg of linearized pSV2Neo in 30 µl H<sub>2</sub>O and 30 µl of 1 M CaCl<sub>2</sub> solution were added. After addition of 60 µl phosphate buffer (50 mM HEPES, 280 mM NaCl, 1.5 mM Na<sub>2</sub> HPO<sub>4</sub>, pH 7.05), the solution was vortexed for 5 seconds, followed by an incubation at room temperature for 25 seconds. The solution was immediately added to 2 ml HP-1 medium containing 2% FCS (2% FCS medium). The medium of an 80% confluent BHK21 cell culture in a 6-well plate was then replaced with the DNA containing medium. After an incubation for 5 hours at 37°C in a CO<sub>2</sub> incubator, the DNA containing medium was removed and replaced by 2 ml of 15% glycerol in 2% FCS medium. The glycerol containing medium was removed after a 30 second incubation phase, and the cells were washed by rinsing with 5 ml of HP-1 medium containing 10% FCS. Finally 2 ml of fresh HP-1 medium containing 10% FCS was added.

[0269] Stably transfected cells were selected and grown in selection medium (HP-1 medium, supplemented with G418) at 37°C in a CO<sub>2</sub> incubator. When the

mixed population was grown to confluency, the culture was split to two dishes, followed by a 12 hours growth period at 37°C. One dish of the cells was shifted to 30°C to induce the expression of the viral particles; the other dish was kept at 37°C.

[0270] The expression of viral particles was determined by Western blotting (Figure 1). Culture medium (0.5 ml) was methanol/chloroform precipitated, and the pellet was resuspended in SDS-PAGE sample buffer. Samples were heated for 5 minutes at 95°C before being applied to 15% acrylamide gel. After SDS-PAGE, proteins were transferred to Protan nitrocellulose membranes (Schleicher & Schuell, Germany) as described by Bass and Yang, in Creighton, T.E., ed., *Protein Function: A Practical Approach*, 2nd Edn., IRL Press, Oxford (1997), pp. 29-55. The membrane was blocked with 1% bovine albumin (Sigma) in TBS (10xTBS per liter: 87.7 g NaCl, 66.1g Trizma hydrochloride (Sigma) and 9.7 g Trizma base (Sigma), pH 7.4) for 1 hour at room temperature, followed by an incubation with an anti-E1/E2 antibody (polyclonal serum) for 1 hour. The blot was washed 3 times for 10 minutes with TBS-T (TBS with 0.05% Tween20), and incubated for 1 hour with an alkaline-phosphatase-anti-rabbit IgG conjugate (0.1 µg/ml, Amersham Life Science, England). After washing 2 times for 10 minutes with TBS-T and 2 times for 10 minutes with TBS, the development reaction was carried out using alkaline phosphatase detection reagents (10 ml AP buffer (100 mM Tris/HCl, 100 mM NaCl, pH 9.5) with 50 µl NBT solution (7.7% Nitro Blue Tetrazolium (Sigma) in 70% dimethylformamide) and 37 µl of X-Phosphate solution (5% of 5-bromo-4-chloro-3-indolyl phosphate in dimethylformamide).

[0271] The production of viral particles is shown in Figure 1. The Western Blot pattern showed that E2-JUN (lane 1) migrated to a higher molecular weight in SDS-PAGE compared to wild type E2 (lane 2) and the BHK21 host cell line did not show any background.

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## EXAMPLE 3:

Production of viral particles containing E2-JUN  
using the pTE5'2JE2:JUN vector

- [0272] RNase-free vector (1.0 µg) was linearized by PvuI digestion. Subsequently, *in vitro* transcription was carried out using an SP6 *in vitro* transcription kit (InvitroscripCAP by InvitroGen, Invitrogen BV, NV Leek, Netherlands). The resulting 5'-capped mRNA was analyzed on a reducing agarose-gel.
- [0273] *In vitro* transcribed mRNA (5 µg) was electroporated into BHK 21 cells (ATCC: CCL10) according to Invitrogen's manual (Sindbis Expression system, Invitrogen BV, Netherlands). After 10 hours incubation at 37°C, the FCS containing medium was exchanged by HP-1 medium without FCS, followed by an additional incubation at 37°C for 10 hours. The supernatant was harvested and analyzed by Western blot analysis for production of viral particles exactly as described in Example 2.
- [0274] The obtained result was identical to the one obtained with pCYTtsE2:JUN as shown in Figure 2.

## EXAMPLE 4:

Fusion of human growth hormone (hGH) to the FOS leucine  
zipper domain (OmpA signal sequence)

- [0275] The hGH gene without the human leader sequence was amplified from the original plasmid (ATCC 31389) by PCR. Oligo 7 with an internal XbaI site was designed for annealing at the 5' end of the hGH gene, and oligo 9 with an internal EcoRI site primed at the 3' end of the hGH gene. For the PCR reaction, 100 pmol of each oligo and 5 ng of the template DNA was used in the 75 µl reaction mixture (4 units of Taq or Pwo polymerase, 0.1 mM dNTPs and 1.5 mM MgSO<sub>4</sub>).
- [0276] PCR cycling was performed in the following manner: 30 cycles with an annealing temperature of 60°C and an elongation time of 1 minute at 72°C.
- [0277] The gel purified and isolated PCR product was used as a template for a second PCR reaction to introduce the ompA signal sequence and the

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Shine-Dalgarno sequence. For the PCR reaction, 100 pmol of oligo 8 and 9 and 1 ng of the template PCR fragment was used in the 75 µl reaction mixture (4 units of Taq or Pwo polymerase, 0.1 mM dNTPs and 1.5 mM MgSO<sub>4</sub>). The annealing temperature for the first five cycles was 55°C with an elongation time of 60 seconds at 72°C; another 25 cycles were performed with an annealing temperature of 65°C and an elongation time of 60 seconds at 72°C.

[0278] *Oligo7*: gggctagattccaaccattcccttatccaggcttttgac aacgctatgctccgcccc atcgtctgcaccagctggcctttgacacc (SEQ ID NO:7); *oligo 8*: gggctagaaggaggtaaaaaa cgatgaaaaagacagctatcgcgattgcagtggcactggctggtttcgctaccgtagcgcaggccttccaac cattcccttatcc (SEQ ID NO:8); *oligo 9*: cccgaattcctagaagccacagctgccctcc (SEQ ID NO:9).

[0279] The resulting recombinant hGH gene was subcloned into pBluescript via XbaI/EcoRI. The correct sequence of both strands was confirmed by DNA sequencing.

[0280] The DNA sequence coding for the *FOS* amphiphatic helix domain was PCR-amplified from vector pJuFo (*Cramer & Suter Gene 137:69* (1993)) using the oligonucleotides:

omp-*FOS*:

5'- ccTGCGGTGGTCTGACCGACACCC-3' (SEQ ID NO:10)

*FOS*-hgh:

5'- ccgcggaagagccaccGCAACCACCGTGTGCCGCCAGGATG-3' (SEQ ID NO:11)

[0281] For the PCR reaction, 100 pmol of each oligo and 5 ng of the template DNA was used in the 75 µl reaction mixture (4 units of Taq or Pwo polymerase, 0.1 mM dNTPs and 1.5 mM MgSO<sub>4</sub>). The temperature cycles were as follows:

[0282] 95°C for 2 minutes, followed by 5 cycles of 95°C (45 seconds), 60°C (30 seconds), 72°C (25 seconds) and followed by 25 cycles of 95°C (45 seconds), 68°C (30 seconds), 72°C (20 seconds).



- [0283] The PCR product was purified, isolated and cloned into the *Stu*I digested pBluescript-ompA-hGH. The hybrid gene was then cloned into the pKK223-3 Plasmid (Pharmacia).

#### EXAMPLE 5:

##### Bacterial expression of FOS-hGH

- [0284] The ompA-*FOS*-hGH in pkk223-3 was expressed under the control of the inducible IPTG-dependend promoter using JM101 as *E. coli* host strain. Expression was performed in shaker flask. Cells were induced with 1 mM IPTG (final concentration) at an OD600 of 0.5. Expression was continued for 10 hours at 37°C. Cells were harvested by centrifugation at 3600 at 10°C for 15 min. The cell pellet was frozen (-20°C or liq. N<sub>2</sub>) and stored for 16 hours. The pellet was then thawed at 4°C and resuspended in 10 ml 10 mM Tris-HCl, pH 7.4 containing 600 mM sucrose. After stirring for 15 min at 4°C, periplasmic proteins were released by an osmotic shock procedure. Chilled (4°C) deionized H<sub>2</sub>O was added, and the suspension was stirred for 30 min at 4°C. The sludge was diluted, resuspended, and lysozyme was added to degrade the cell wall of the bacteria. The cells and the periplasmic fraction spheroplasts were separated by centrifugation for 20 min at 11000 x g at 4°C. The *FOS*-hGH-containing supernatant was analyzed by reducing and non-reducing SDS-Page and Dot Blot. Dot Blot was carried out as described in Example 8, using an anti-hGH antibody (Sigma) as the first antibody and an alkaline phosphatase (AP)-anti-mouse antibody conjugate as the second antibody.
- [0285] Full length, correctly processed *FOS*-hGH could be detected under reducing and non-reducing conditions. Part of *FOS*-hGH was bound to other, non-identified proteins due to the free cysteines present in the *FOS* amphiphatic helix. However, more than 50% of expressed *FOS*-hGH occurred in its native monomeric conformation ( Figure 3).
- [0286] Purified *FOS*-hGH will be used to perform first doping experiments with *JUN* containing viral particles.

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## EXAMPLE 6:

Construction of the pAV vector series for expression of FOS fusion proteins

[0287] A versatile vector system was constructed that allowed either cytoplasmic production or secretion of N- or C-terminal *FOS* fusion proteins in *E. coli* or production of N- or C-terminal *FOS* fusion proteins in eukaryotic cells. The vectors pAV1 - pAV4 which was designed for production of *FOS* fusion proteins in *E. coli*, encompasses the DNA cassettes listed below, which contain the following genetic elements arranged in different orders: (a) a strong ribosome binding site and 5'-untranslated region derived from the *E. coli* ompA gene (aggaggtaaaaaacg) (SEQ ID NO:13); (b) a sequence encoding the signal peptide of *E. coli* outer membrane protein OmpA (MKKTAIAIAVALAGFATVAQA) (SEQ ID NO:14); (c) a sequence coding for the *FOS* dimerization domain flanked on both sides by two glycine residues and a cystein residue (CGGLTDTLQAETDQVEDEKSALQTEIANLLKEKEKLEFILAAHGGC) (SEQ ID NO:15); and (d) a region encoding a short peptidic linker (AAASGG (SEQ ID NO:16) or GGSAAA (SEQ ID NO:17)) connecting the protein of interest to the *FOS* dimerization domain. Relevant coding regions are given in upper case letters. The arrangement of restriction cleavage sites allows easy construction of *FOS* fusion genes with or without a signal sequence. The cassettes are cloned into the EcoRI/HindIII restriction sites of expression vector pKK223-3 (Pharmacia) for expression of the fusion genes under control of the strong tac promotor.

pAV1

[0288] This vector was designed for the secretion of fusion proteins with *FOS* at the C-terminus into the *E. coli* periplasmic space. The gene of interest (g.o.i.) may be ligated into the StuI/NotI sites of the vector.

EcoRI

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gaa ttc agg agg taa aaa acg ATG AAA AAG ACA GCT ATC GCG ATT GCA  
GTG GCA CTG GCT

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                                M   K   K   T   A   I   A   I   A
V   A   L   A

61/21                               StuI                               NotI

GGT TTC GCT ACC GTA GCG CAG GCC tgg gtg ggg GCG GCC GCT TCT GGT
GGT TGC GGT GGT
G   F   A   T   V   A   Q   A   (goi)       A   A   A   S   G
G   C   G   G

121/41                               151/51

CTG ACC GAC ACC CTG CAG GCG GAA ACC GAC CAG GTG GAA GAC GAA AAA
TCC GCG CTG CAA
L   T   D   T   L   Q   A   E   T   D   Q   V   E   D   E   K
S   A   L   Q

181/61                               211/71

ACC GAA ATC GCG AAC CTG CTG AAA GAA AAA GAA AAG CTG GAG TTC ATC
CTG GCG GCA CAC
T   E   I   A   N   L   L   K   E   K   E   K   L   E   F   I
L   A   A   H

241/81           HindIII
GGT GGT TGC taa gct t   (SEQ ID NO:18)
G   G   C   *   A       (SEQ ID NOs:14 and 19)

```

pAV2

[0289] This vector was designed for the secretion of fusion proteins with *FOS* at the N-terminus into the *E. coli* periplasmic space. The gene of interest (g.o.i.) ligated into the NotI/EcoRV (or NotI/HindIII) sites of the vector.

```

EcoRI                               31/11

gaa ttc agg agg taa aaa acg ATG AAA AAG ACA GCT ATC GCG ATT GCA
GTG GCA CTG GCT
                                M   K   K   T   A   I   A   I   A
V   A   L   A

61/21                               StuI                               91/31

GGT TTC GCT ACC GTA GCG CAG GCC TGC GGT GGT CTG ACC GAC ACC CTG
CAG GCG GAA ACC
G   F   A   T   V   A   Q   A   C   G   G   L   T   D   T   L
Q   A   E   T

```

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121/41

151/51

GAC CAG GTG GAA GAC GAA AAA TCC GCG CTG CAA ACC GAA ATC GCG AAC  
 CTG CTG AAA GAA  
 D Q V E D E K S A L Q T E I A N  
 L L K E

181/61

211/71

NotI  
 AAA GAA AAG CTG GAG TTC ATC CTG GCG GCA CAC GGT GGT TGC GGT GGT  
 TCT GCG GCC GCT  
 K E K L E F I L A A H G G C G G  
 S A A A

241/81

EcoRV HindIII

ggg tgt ggg gat atc aaq ctt (SEQ ID NO:20)  
 (goi) (SEQ ID NO:21)

pAV3

[0290] This vector was designed for the cytoplasmic production of fusion proteins with *FOS* at the C-terminus in *E. coli*. The gene of interest (g.o.i.) may be ligated into the EcoRV/NotI sites of the vector.

EcoRI

EcoRV

NotI

gaa ttc agg agg taa aaa gat atc ggg tgt ggg GCG GCC GCT TCT GGT  
 GGT TGC GGT GGT  
 (goi) A A A S G  
 G C G G

61/21

91/31

CTG ACC GAC ACC CTG CAG GCG GAA ACC GAC CAG GTG GAA GAC GAA AAA  
 TCC GCG CTG CAA  
 L T D T L Q A E T D Q V E D E K  
 S A L Q

121/41

151/51

ACC GAA ATC GCG AAC CTG CTG AAA GAA AAA GAA AAG CTG GAG TTC ATC  
 CTG GCG GCA CAC  
 T E I A N L L K E K E K L E F I  
 L A A H

181/61

HindIII

GGT GGT TGC taa gct t (SEQ ID NO:22)  
 G G C \* (SEQ ID NO:23)

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pAV4

[0291] This vector is designed for the cytoplasmic production of fusion proteins with *FOS* at the N-terminus in *E. coli*. The gene of interest (g.o.i.) may be ligated into the NotI/EcoRV (or NotI/HindIII) sites of the vector. The N-terminal methionine residue is proteolytically removed upon protein synthesis (Hirel *et al.*, *Proc. Natl. Acad. Sci. USA* 86:8247-8251 (1989)).

EcoRI

31/11

gaa ttc agg agg taa aaa acg ATG GCT TGC GGT GGT CTG ACC GAC ACC  
 CTG CAG GCG GAA  
 E F R R \* K T M A C G G L T D T  
 L Q A E

61/21

91/31

ACC GAC CAG GTG GAA GAC GAA AAA TCC GCG CTG CAA ACC GAA ATC GCG  
 AAC CTG CTG AAA  
 T D Q V E D E K S A L Q T E I A  
 N L L K

121/41

151/51

NotI  
 GAA AAA GAA AAG CTG GAG TTC ATC CTG GCG GCA CAC GGT GGT TGC GGT  
 GGT TCT GCG GCC  
 E K E K L E F I L A A H G G C G  
 G S A A

181/61

EcoRV HindIII

GCT ggg tgt ggg gat atc aag ctt (SEQ ID NO:24)  
 A (goi) (SEQ ID NOs:88 and 25)

[0292] The vectors pAV5 and pAV6, which are designed for eukaryotic production of *FOS* fusion proteins, encompasses the following genetic elements arranged in different orders: (a) a region coding for the leader peptide of human growth hormone (MATGSRTSLLLAFLCLPWLQEGSA) (SEQ ID NO:26); (b) a sequence coding for the *FOS* dimerization domain flanked on both sides by two glycine residues and a cysteine residue (CGGLTDTLQAETDQVEDEKSALQTEIANLLKEKEKLEFILAAHGGC) (SEQ ID NO:15); and

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(c) a region encoding a short peptidic linker (AAASGG (SEQ ID NO:16) or GGSAAA (SEQ ID NO:17)) connecting the protein of interest to the *FOS* dimerization domain. Relevant coding regions are given in upper case letters. The arrangement of restriction cleavage sites allows easy construction of *FOS* fusion genes. The cassettes are cloned into the EcoRI/HindIII restriction sites of the expression vector pMPSVEH (Artelt *et al.*, *Gene* 68:213-219 (1988)).

### pAV5

[0293] This vector is designed for the eukaryotic production of fusion proteins with *FOS* at the C-terminus. The gene of interest (g.o.i.) may be inserted between the sequences coding for the hGH signal sequence and the *FOS* domain by ligation into the Eco47III/NotI sites of the vector. Alternatively, a gene containing its own signal sequence may be fused to the *FOS* coding region by ligation into the StuI/NotI sites.

```

EcoRI   StuI                               31/11

gaa ttc agg cct ATG GCT ACA GGC TCC CGG ACG TCC CTG CTC CTG GCT
TTT GGC CTG CTC
      M  A  T  G  S  R  T  S  L  L  L  A
F   G   L   L

61/21                               Eco47III           NotI

TGC CTG CCC TGG CTT CAA GAG GGC AGC GCT ggg tgt ggg GCG GCC GCT
TCT GGT GGT TGC
C   L   P   W   L   Q   E   G   S   A   (goi)   A   A   A
S   G   G   C

121/41                               151/51

GGT GGT CTG ACC GAC ACC CTG CAG GCG GAA ACC GAC CAG GTG GAA GAC
GAA AAA TCC GCG
G   G   L   T   D   T   L   Q   A   E   T   D   Q   V   E   D
E   K   S   A

181/61                               211/71

CTG CAA ACC GAA ATC GCG AAC CTG CTG AAA GAA AAA GAA AAG CTG GAG
TTC ATC CTG GCG
L   Q   T   E   I   A   N   L   L   K   E   K   E   K   L   E
F   I   L   A

```

**[0294]** This vector is designed for the eukaryotic production of fusion proteins with *FOS* at the N-terminus. The gene of interest (g.o.i.) may be ligated into the NotI/StuI (or NotI/HindIII) sites of the vector.

61/21						Eco47III	91/31							
CCC TGG CTT CAA GAG GGC	<u>AGC GCT</u>	TGC GGT GGT CTG ACC GAC ACC CTG												
CAG GCG GAA ACC														
P W L Q E G S A C G G L T D T L														
Q A E T														

[illegible][illegible]

241/81      StuI      HindIII  
ggg tgt ggg agg cct aaq ctt      (SEQ ID NO:29)  
(goi)      (SEQ ID NO:30)

## Construction of expression vectors pAV1 - pAV6

[0295] The following oligonucleotides have been synthesized for construction of expression vectors pAV1 - pAV6:

*FOS*-FOR1:

CCTGGGTGGGGGCGGCCGCTTCTGGTGGTTGCGGTGGTCTGACC (SEQ ID NO:31);

*FOS*-FOR2:

GGTGGGAATTCAGGAGGTAAAAAGATATCGGGTGTGGGGCGGCC (SEQ ID NO:32);

*FOS*-FOR3:

GGTGGGAATTCAGGAGGTAAAAACGATGGCTTGCGGTGGTCTGACC (SEQ ID NO:33);

*FOS*-FOR4:

GCTTGCGGTGGTCTGACC (SEQ ID NO:34);

*FOS*-REV1:

CCACCAAGCTTAGCAACCACCGTGTGC (SEQ ID NO:35);

*FOS*-REV2:

CCACCAAGCTTGATATCCCCACACCCAGCGGCCGCAGAACCACCGC AACCACCG (SEQ ID NO:36);

*FOS*-REV3:

CCACCAAGCTTAGGCCTCCCACACCCAGCGGC (SEQ ID NO:37);

*OmpA*-FOR1:

GGTGGGAATTCAGGAGGTAAAAACGATG (SEQ ID NO:38);

*hGH*-FOR1:

GGTGGGAATTCAGGCCTATGGCTACAGGCTCC (SEQ ID NO:39); and

*hGH*-FOR2:

GGTGGGAATTCATGGCTACAGGCTCCC (SEQ ID NO:40).



- [0296] For the construction of vector pAV2, the regions coding for the OmpA signal sequence and the *FOS* domain were amplified from the ompA-*FOS*-hGH fusion gene in vector pKK223-3 (*see* Example 5) using the primer pair OmpA-FOR1/ *FOS*-REV2. The PCR product was digested with EcoRI/HindIII and ligated into the same sites of vector pKK223-3 (Pharmacia).
- [0297] For the construction of vector pAV1, the *FOS* coding region was amplified from the ompA-*FOS*-hGH fusion gene in vector pKK223-3 (*see* Example 5) using the primer pair *FOS*-FOR1/ *FOS*-REV1. The PCR product was digested with HindIII and ligated into StuI/HindIII digested vector pAV2.
- [0298] For the construction of vector pAV3, the region coding for the *FOS* domain was amplified from vector pAV1 using the primer pair *FOS*-FOR2/*FOS*-REV1. The PCR product was digested with EcoRI/HindIII and ligated into the same sites of the vector pKK223-3 (Pharmacia).
- [0299] For the construction of vector pAV4, the region coding for the *FOS* domain was amplified from the ompA-*FOS*-hGH fusion gene in vector pKK223-3 (*see* Example 5) using the primer pair *FOS*-FOR3/*FOS*-REV2. The PCR product was digested with EcoRI/HindIII and ligated into the same sites of the vector pKK223-3 (Pharmacia).
- [0300] For the construction of vector pAV5, the region coding for the hGH signal sequence is amplified from the hGH-*FOS*-hGH fusion gene in vector pSINrep5 (*see* Example 7) using the primer pair hGH-FOR1/hGHREV1. The PCR product is digested with EcoRI/NotI and ligated into the same sites of the vector pAV1. The resulting cassette encoding the hGH signal sequence and the *FOS* domain is then isolated by EcoRI/HindIII digestion and cloned into vector pMPSVEH (Artelt *et al.*, *Gene* 68:213-219 (1988)) digested with the same enzymes.
- [0301] For the construction of vector pAV6, the *FOS* coding region is amplified from vector pAV2 using the primer pair *FOS*-FOR4/*FOS*REV3. The PCR product is digested with HindIII and cloned into Eco47III/HindIII cleaved vector pAV5. The entire cassette encoding the hGH signal sequence and the *FOS* domain is then reamplified from the resulting vector using the primer pair

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hGH-FOR2/*FOS*REV3, cleaved with EcoRI/HindIII and ligated into vector pMPSVEH (Artelt *et al.*, *Gene* 68:213-219 (1988)) cleaved with the same enzymes.

#### EXAMPLE 7:

##### Construction of FOS-hGH with human (hGH) signal sequence

[0302] For eukaryotic expression of the *FOS*-hGH fusion protein, the *OmpA-FOS*-hGH fusion gene was isolated from pBluescript::OmpA-*FOS*-hGH (see Example 4) by digestion with XbaI/Bsp120I and cloned into vector pSINrep5 (Invitrogen) cleaved with the same enzymes. The hGH signal sequence was synthesized by PCR (reaction mix: 50 pmol of each primer, dATP, dGTP, dTTP, dCTP (200 µM each), 2.5 U Taq DNA polymerase (Qiagen), 50 µl total volume in the buffer supplied by the manufacturer; amplification: 92°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, 30 cycles) using the overlapping oligonucleotides Sig-hGH-FOR (GGGTCTAGAAATGGCTACAGGCTCCCGGACGTCCTGCTCCTGGCTT TTGGCCTGCTCTG) (SEQ ID NO:41) and Sig-hGH-REV (CGCAGGCCTCGGCACTGCCCTCTTGAAGCCAGGGCAGGCAGAGCA GGCCAAAAGCCAG) (SEQ ID NO:42). The PCR product was purified using the QiaExII Kit, digested with StuI/XbaI and ligated into vector pSINrep5::OmpA-*FOS*-hGH cleaved with the same enzymes.

#### EXAMPLE 8:

##### Eukaryotic expression of FOS-hGH

[0303] RNase-free vector (1.0 µg) (pSINrep5::OmpA-*FOS*-hGH) and 1.0 µg of DHEB (Bredenbeek *et al.*, *J. Virol.* 67:6439-6446 (1993)) were linearized by ScaI restriction digest. Subsequently, *in vitro* transcription was carried out using an SP6 *in vitro* transcription kit (InvitroscipCAP by InvitroGen, Invitrogen BV, NV Leek, Netherlands). The resulting 5'-capped mRNA was analyzed on reducing agarose-gel.

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[0304] *In vitro*, transcribed mRNA 5 µg was electroporated into BHK 21 cells (ATCC: CCL10) according to Invitrogen's manual (Sindbis Expression system, Invitrogen BV, Netherlands). After 10 hours incubation at 37°C the FCS containing medium was exchanged by HP-1 medium without FCS, followed by an additional incubation at 37°C for 10 hours. The supernatant was harvested and analyzed by dot-blot analysis for production of *FOS*-hgh.

[0305] Culture media (2.5 µl) was spotted on a nitrocellulose membrane and dried for 10 minutes at room temperature. The membrane was blocked with 1 % bovine albumin (Sigma) in TBS (10xTBS per liter: 87.7 g NaCl, 66.1g Trizma hydrochloride (Sigma) and 9.7 g Trizma base (Sigma), pH 7.4) for 1 hour at room temperature, followed by an incubation with 2 µg rabbit anti-human hGH antibody (Sigma) in 10 ml TBS-T (TBS with 0.05% Tween20) for 1 hour. The blot was washed 3 times for 10 minutes with TBS-T and incubated for 1 hour with alkaline phosphatase conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:5000 in TBS-T. After washing 2 times for 10 minutes with TBS-T and 2 times for 10 minutes with TBS, the blot was developed by AP staining as described in Example 2. Results are shown in Figure 3.

#### EXAMPLE 9:

##### Construction of FOS-PLA (N- and C-terminal)

[0306] The following gene is constructed by chemical gene synthesis coding for a catalytically inactive variant (Förster *et al.*, *J. Allergy Clin. Immunol.* 95: 1229-1235 (1995)) of bee venom phospholipase A<sub>2</sub> (PLA).

1/1

31/11

```

ATC ATC TAC CCA GGT ACT CTG TGG TGT GGT CAC GGC AAC AAA TCT TCT
GGT CCG AAC GAA
I   I   Y   P   G   T   L   W   C   G   H   G   N   K   S   S
G   P   N   E

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91/31

```

CTC GGC CGC TTT AAA CAC ACC GAC GCA TGC TGT CGC ACC CAG GAC ATG
TGT CCG GAC GTC
L   G   R   F   K   H   T   D   A   C   C   R   T   Q   D   M
C   P   D   V

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ATG TCT GCT GGT GAA TCT AAA CAC GGG TTA ACT AAC ACC GCT TCT CAC  
 ACG CGT CTC AGC  
 M S A G E S K H G L T N T A S H  
 T R L S

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211/71

TGC GAC TGC GAC GAC AAA TTC TAC GAC TGC CTT AAG AAC TCC GCC GAT  
 ACC ATC TCT TCT  
 C D C D D K F Y D C L K N S A D  
 T I S S

241/81

271/91

TAC TTC GTT GGT AAA ATG TAT TTC AAC CTG ATC GAT ACC AAA TGT TAC  
 AAA CTG GAA CAC  
 Y F V G K M Y F N L I D T K C Y  
 K L E H

301/101

331/111

CCG GTA ACC GGC TGC GGC GAA CGT ACC GAA GGT CGC TGC CTG CAC TAC  
 ACC GTT GAC AAA  
 P V T G C G E R T E G R C L H Y  
 T V D K

361/121

391/131

TCT AAA CCG AAA GTT TAC CAG TGG TTC GAC CTG CGC AAA TAC (SEQ  
 ID NO:43)  
 S K P K V Y Q W F D L R K Y (SEQ  
 ID NO:44)

[0307] For fusion of PLA to the N-terminus of the *FOS* dimerization domain, the region is amplified using the oligonucleotides PLA-FOR1 (CCATCATCTACCCAGGTAC) (SEQ ID NO:45) and PLA-REV1 (CCCACACCCAGCGGCCGCGTATTTGCGCAGGTCG) (SEQ ID NO:46). The PCR product is cleaved with NotI and ligated into vector pAV1 previously cleaved with the restriction enzymes StuI/NotI. For fusion of PLA to the C-terminus of the *FOS* dimerization domain, the region is amplified using the oligonucleotides PLA-FOR2 (CGGTGGTTCTGCGGCCGCTATCATCTACCCAGGTAC) (SEQ ID NO:47) and PLA-REV2 (TTAGTATTTGCGCAGGTCG) (SEQ ID NO:48). The PCR

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product is cleaved with NotI and ligated into vector pAV2 previously cleaved with the restriction enzymes NotI/EcoRV.

#### EXAMPLE 10:

##### Construction of FOS-Ovalbumin fusion gene (N- and C-terminal)

[0308] For cloning of the ovalbumin coding sequence, mRNA from chicken oviduct tissue is prepared using the QuickPrep™ Micro mRNA Purification Kit (Pharmacia) according to manufacturer instructions. Using the SuperScript™ One-step RT PCR Kit (Gibco BRL), a cDNA encoding the mature part of ovalbumin (corresponding to nucleotides 68-1222 of the mRNA (McReynolds *et al.*, *Nature* 273:723-728 (1978)) is synthesized using the primers Ova-FOR1 (CCGGCTCCATCGGTGCAG) (SEQ ID NO:49) and Ova-REV1 (ACCACCAGAAGCGGCCGCAGGGGAAACACATCTGCC) (SEQ ID NO:50). The PCR product is digested with NotI and cloned into StuI/NotI digested vector pAV1 for expression of the fusion protein with the *FOS* dimerization domain at the C terminus. For production of a fusion protein with the *FOS* dimerization domain at the N terminus, the Ovalbumin coding region is amplified from the constructed vector (pAV1::Ova) using the primers Ova-FOR2 (CGGTGGTTCTGCGGCCGCTGGCTCCATCGGTGCAG) (SEQ ID NO:51) and Ova-REV2 (TTAAGGGGAAACACATCTGCC) (SEQ ID NO:52). The PCR product is digested with NotI and cloned into the NotI/EcoRV digested vector pAV2. Cloned fragments are verified by DNA sequence analysis.

#### EXAMPLE 11

##### Production and purification of FOS-PLA and

##### FOS ovalbumin fusion proteins

[0309] For cytoplasmic production of *FOS* fusion proteins, an appropriate *E. coli* strain was transformed with the vectors pAV3::PLA, pAV4::PLA, pAV3::Ova or pAV4::Ova. The culture was incubated in rich medium in the presence of ampicillin at 37°C with shaking. At an optical density (550nm) of 1, 1 mM IPTG

was added and incubation was continued for another 5 hours. The cells were harvested by centrifugation, resuspended in an appropriate buffer (e.g., tris-HCl, pH 7.2, 150 mM NaCl) containing DNase, RNase and lysozyme, and disrupted by passage through a french pressure cell. After centrifugation (Sorvall RC-5C, SS34 rotor, 15000 rpm, 10 min, 4°C), the pellet was resuspended in 25 ml inclusion body wash buffer (20 mM tris-HCl, 23% sucrose, 0.5% Triton X-100, 1 mM EDTA, pH8) at 4°C and recentrifuged as described above. This procedure was repeated until the supernatant after centrifugation was essentially clear. Inclusion bodies were resuspended in 20 ml solubilization buffer (5.5 M guanidinium hydrochloride, 25 mM tris-HCl, pH 7.5) at room temperature and insoluble material was removed by centrifugation and subsequent passage of the supernatant through a sterile filter (0.45 µm). The protein solution was kept at 4°C for at least 10 hours in the presence of 10 mM EDTA and 100 mM DTT and then dialyzed three times against 10 volumes of 5.5 M guanidinium hydrochloride, 25 mM tris-HCl, 10 mM EDTA, pH 6. The solution was dialyzed twice against 5 liters of 2 M urea, 4 mM EDTA, 0.1 M NH<sub>4</sub>Cl, 20 mM sodium borate (pH 8.3) in the presence of an appropriate redox shuffle (oxidized glutathione/reduced glutathione; cystine/cysteine). The refolded protein was then applied to an ion exchange chromatography. The protein was stored in an appropriate buffer with a pH above 7 in the presence of 2-10 mM DTT to keep the cysteine residues flanking the *FOS* domain in a reduced form. Prior to coupling of the protein with the alphavirus particles, DTT was removed by passage of the protein solution through a Sephadex G-25 gel filtration column.

#### EXAMPLE 12:

##### Constructions of gp140-FOS

[0310] jThe gp140 gene (Swiss-Prot:P03375) without the internal protease cleavage site was amplified by PCR from the original plasmid pAbT4674 (ATCC 40829) containing the full length gp160 gene using the following oligonucleotides:

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HIV-1:

5'-ACTAGTCTAGAatgagagtgaaggagaaatc-3' (SEQ ID NO:53);

HIV-end:

5'-TAGCATGCTAGCACCGAAttatctaattccaataattcttg-3' (SEQ ID NO:54);

HIV-Cleav:

5'-gtagcaccacccaaggcaaagCTGAAAGCTACCCAGCTCGAGAAACTGgca-3'  
(SEQ ID NO:55); and

HIV-Cleav2:

5'-caaagctcctattcccactgcCAGTTTCTCGAGCTGGGTAGCTTTCAG-3'  
(SEQ ID NO:56).

[0311] For PCR I, 100 pmol of oligo HIV-1 and HIV-Cleav2 and 5 ng of the template DNA were used in the 75  $\mu$ l reaction mixture (4 units of Taq or Pwo polymerase, 0.1 mM dNTPs and 1.5 mM MgSO<sub>4</sub>). PCR cycling was done in the following manner: 30 cycles with an annealing temperature of 60°C and an elongation time of 2 minutes at 72°C.

[0312] For PCR II, 100 pmol of oligo HIV-end and HIV-Cleav and 5 ng of the template DNA were used in the 75  $\mu$ l reaction mixture, (4 units of Taq or Pwo polymerase, 0.1 mM dNTPs and 1.5 mM MgSO<sub>4</sub>). PCR cycling was done in the following manner: 30 cycles with an annealing temperature of 60°C and an elongation time of 50 seconds at 72°C.

[0313] Both PCR fragments were purified, isolated and used in an assembly PCR reaction. For the assembly PCR reaction, 100 pmol of oligo HIV-1 and HIV-end and 2 ng of each PCR fragment (PCR I and PCR II) were used in the 75  $\mu$ l (4 units of Taq or Pwo polymerase, 0.1 mM dNTPs and 1.5 mM MgSO<sub>4</sub>). PCR cycling was done in the following manner: 30 cycles with an annealing temperature of 60°C and an elongation time of 2.5 minutes at 72°C. The assembly PCR product was digested *Xba*I and *Nhe*I. The *FOS* amphipathic helix was fused in frame to the C-terminal end of gp-140.

[0314] The DNA sequence coding for the *FOS* amphiphatic helix domain was PCR-amplified from vector pJuFo (Cramer & Suter *Gene* 137:69 (1993)) using the oligonucleotides:

*FOS*-HIV:

5'-ttcgggtgctagcgggtggcTGCGGTGGTCTGACCGAC-3' (SEQ ID NO:57); and

*FOS*-Apa:

5'-gatgctggggccctaaccGCAACCACCGTGTGCCGCC-3' (SEQ ID NO:58).

[0315] For the PCR reaction, 100 pmol of each oligo and 5 ng of the template DNA was used in the 75 µl reaction mixture (4 units of Taq or Pwo polymerase, 0.1 mM dNTPs and 1.5 mM MgSO<sub>4</sub>). Temperature cycling was done as follows: 95°C for 2 minutes, followed by 5 cycles of 95°C (45 seconds), 60°C (30 seconds), 72°C (25 seconds) and followed by 25 cycles of 95°C (45 seconds), 68°C (30 seconds), 72°C (20 seconds). The obtained PCR fragment was digested with NheI and Bsp120L.

[0316] The final expression vector for GP140-*FOS* was obtained in a 3 fragment ligation of both PCR fragments into pSinRep5. The resultant vector pSinRep5-GP140-*FOS* was evaluated by restriction analysis and DNA sequencing.

[0317] GP140-*FOS* was also cloned into pCYTts via XbaI and Bsp120L to obtain a stable, inducible GP140-*FOS* expressing cell line.

#### EXAMPLE 13:

##### Expression of GP140FOS using pSinRep5-GP140FOS

[0318] RNase-free vector (1.0 µg) (pSinRep5-GP140-*FOS*) and 1.0 µg of DHEB (Bredenbeek *et al.*, *J. Virol.* 67:6439-6446 (1993)) were linearized by restriction digestion. Subsequently, *in vitro* transcription was carried out using an SP6 *in vitro* transcription kit (InvitroscriptCAP by InvitroGen, Invitrogen BV, NV Leek, Netherlands). The resulting 5'-capped mRNA was analyzed on a reducing agarose-gel.

[0319] *In vitro* transcribed mRNA (5 µg) was electroporated into BHK 21 cells (ATCC: CCL10) according to Invitrogen's manual (Sindbis Expression System,



Invitrogen BV, Netherlands). After 10 hours incubation at 37°C, the FCS containing medium was exchanged by HP-1 medium without FCS, followed by an additional incubation at 37°C for 10 hours. The supernatant was harvested and analyzed by Western blot analysis for production of soluble GP140-*FOS* exactly as described in Example 2.

#### EXAMPLE 14:

##### Expression of GP140FOS using pCYTts-GP140FOS

[0320] pCYT-GP140-*FOS* 20 µg was linearized by restriction digestion. The reaction was stopped by phenol/chloroform extraction, followed by an isopropanol precipitation of the linearized DNA. The restriction digestion was evaluated by agarose gel electrophoresis. For the transfection, 5.4 µg of linearized pCYTtsGP140-*FOS* was mixed with 0.6 µg of linearized pSV2Neo in 30 µl H<sub>2</sub>O and 30 µl of 1 M CaCl<sub>2</sub> solution was added. After addition of 60 µl phosphate buffer (50 mM HEPES, 280 mM NaCl, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.05), the solution was vortexed for 5 seconds, followed by an incubation at room temperature for 25 seconds. The solution was immediately added to 2 ml HP-1 medium containing 2% FCS (2% FCS medium). The medium of an 80% confluent BHK21 cell culture (6-well plate) was then replaced by the DNA containing medium. After an incubation for 5 hours at 37°C in a CO<sub>2</sub> incubator, the DNA containing medium was removed and replaced by 2 ml of 15% glycerol in 2% FCS medium. The glycerol containing medium was removed after a 30 second incubation phase, and the cells were washed by rinsing with 5 ml of HP-1 medium containing 10% FCS. Finally 2 ml of fresh HP-1 medium containing 10% FCS was added.

[0321] Stably transfected cells were selected and grown in selection medium (HP-1 medium supplemented with G418) at 37°C in a CO<sub>2</sub> incubator. When the mixed population was grown to confluency, the culture was split to two dishes, followed by a 12 h growth period at 37°C. One dish of the cells was shifted to

30°C to induce the expression of soluble GP140-*FOS*. The other dish was kept at 37°C.

[0322] The expression of soluble GP140-*FOS* was determined by Western blot analysis. Culture media (0.5 ml) was methanol/chloroform precipitated, and the pellet was resuspended in SDS-PAGE sample buffer. Samples were heated for 5 minutes at 95°C before being applied to a 15% acrylamide gel. After SDS-PAGE, proteins were transferred to Protan nitrocellulose membranes (Schleicher & Schuell, Germany) as described by Bass and Yang, in Creighton, T.E., ed., *Protein Function: A Practical Approach*, 2nd Edn., IRL Press, Oxford (1997), pp. 29-55. The membrane was blocked with 1 % bovine albumin (Sigma) in TBS (10xTBS per liter: 87.7 g NaCl, 66.1g Trizma hydrochloride (Sigma) and 9.7 g Trizma base (Sigma), pH 7.4) for 1 hour at room temperature, followed by an incubation with an anti-GP140 or GP-160 antibody for 1 hour. The blot was washed 3 times for 10 minutes with TBS-T (TBS with 0.05% Tween20), and incubated for 1 hour with an alkaline-phosphatase-anti-mouse/rabbit/monkey/human IgG conjugate. After washing 2 times for 10 minutes with TBS-T and 2 times for 10 minutes with TBS, the development reaction was carried out using alkaline phosphatase detection reagents (10 ml AP buffer (100 mM Tris/HCl, 100 mM NaCl, pH 9.5) with 50 µl NBT solution (7.7% Nitro Blue Tetrazolium (Sigma) in 70% dimethylformamide) and 37 µl of X-Phosphate solution (5% of 5-bromo-4-chloro-3-indolyl phosphate in dimethylformamide).

#### EXAMPLE 15:

##### Production and purification of GP140FOS

[0323] An anti-gp120 antibody was covalently coupled to a NHS/EDC activated dextran and packed into a chromatography column. The supernatant, containing GP140FOS is loaded onto the column and after sufficient washing, GP140FOS was eluted using 0.1 M HCl. The eluate was directly neutralized during collection using 1 M Tris pH 7.2 in the collection tubes.

[0324] Disulfide bond formation might occur during purification, therefore the collected sample is treated with 10 mM DTT in 10 mM Tris pH 7.5 for 2 hours at 25°C.

[0325] DTT is removed by subsequent dialysis against 10 mM Mes; 80 mM NaCl pH 6.0. Finally GP140FOS is mixed with alphavirus particles containing the *JUN* leucine zipper in E2 as described in Example 16.

#### EXAMPLE 16:

##### Preparation of the AlphaVaccine Particles

[0326] Viral particles (*see* Examples 2 and 3) were concentrated using Millipore Ultrafree Centrifugal Filter Devices with a molecular weight cut-off of 100 kD according to the protocol supplied by the manufacturer. Alternatively, viral particles were concentrated by sucrose gradient centrifugation as described in the instruction manual of the Sindbis Expression System (Invitrogen, San Diego, California). The pH of the virus suspension was adjusted to 7.5 and viral particles were incubated in the presence of 2-10 mM DTT for several hours. Viral particles were purified from contaminating protein on a Sephacryl S-300 column (Pharmacia) (viral particles elute with the void volume) in an appropriate buffer.

[0327] Purified virus particles were incubated with at least 240 fold molar excess of FOS-antigen fusion protein in an appropriate buffer (pH 7.5-8.5) in the presence of a redox shuffle (oxidized glutathione/reduced glutathione; cystine/cysteine) for at least 10 hours at 4°C. After concentration of the particles using a Millipore Ultrafree Centrifugal Filter Device with a molecular weight cut-off of 100 kD, the mixture was passed through a Sephacryl S-300 gel filtration column (Pharmacia). Viral particles were eluted with the void volume.

## EXAMPLE 17:

Fusion of JUN amphipathic helix to the amino terminus of HBcAg(1-144)

[0328] The JUN helix was fused to the amino terminus of the HBcAg amino acid sequence 1 to 144 (JUN-HBcAg construct). For construction of the JUN-HBcAg DNA sequence, the sequences encoding the JUN helix and HBcAg(1-144) were amplified separately by PCR. The JUN sequence was amplified from the pJuFo plasmid using primers EcoRI-JUN(s) and JUN-SacII(as). The EcoRI-JUN(s) primer introduced an EcoRI site followed by a start ATG codon. The JUN-SacII(as) primer introduced a linker encoding the amino acid sequence GAAGS. The HBcAg (1-144) sequence was amplified from the pEco63 plasmid (obtained from ATCC No. 31518) using primers JUN-HBcAg(s) and HBcAg(1-144)Hind(as). JUN-HBcAg(s) contained a sequence corresponding to the 3' end of the sequence encoding the JUN helix followed by a sequence encoding the GAAGS linker and the 5' end of the HBcAg sequence. HBcAg(1-144)Hind(as) introduces a stop codon and a HindIII site after codon 144 of the HBcAg gene. For the PCR reactions, 100 pmol of each oligo and 50 ng of the template DNAs were used in the 50 µl reaction mixtures with 2 units of Pwo polymerase, 0.1 mM dNTPs and 2 mM MgSO<sub>4</sub>. For both reactions, temperature cycling was carried out as follows: 94°C for 2 minutes; and 30 cycles of 94°C (1 minute), 50°C (1 minute), 72°C (2 minutes).

[0329] Primer sequences:

EcoRI-JUN(s):

(5'-CCGGAATTCATGTGCGGTGGTCGGATCGCCCGG-3') (SEQ ID NO:61);

JUN-SacII(as):

(5'-GTCGCTACCCGCGGCTCCGCAACCAACGTGGTTCATGAC-3')(SEQ ID NO:62);

JUN-HBcAg(s):

(5'-GTTGGTTGCGGAGCCGCGGGTAGCGACATTGACCCTTATAAAGAATTTGG-3')  
(SEQ ID NO:63);

HBcAg(1-144)Hind(as):

(5'-CGCGTCCCAAGCTTCTACGGAAGCGTTGATAGGATAGG-3') (SEQ  
ID NO:64).

[0330] Fusion of the two PCR fragments was performed by PCR using primers EcoRI-JUN(s) and HBcAg(1-144)Hind(as). 100 pmol of each oligo was used with 100ng of the purified PCR fragments in a 50 µl reaction mixture containing 2 units of Pwo polymerase, 0.1 mM dNTPs and 2 mM MgSO<sub>4</sub>. PCR cycling conditions were: 94°C for 2 minutes; and 35 cycles of 94°C (1 minute), 50°C (1 minute), 72°C (2 minutes). The final PCR product was analyzed by agarose gel electrophoresis, purified and digested for 16 hours in an appropriate buffer with EcoRI and HindIII restriction enzymes. The digested DNA fragment was ligated into EcoRI/HindIII-digested pKK vector to generate pKK-JUN-HBcAg expression vector. Insertion of the PCR product was analyzed by EcoRI/HindIII restriction analysis and by DNA sequencing of the insert.

#### EXAMPLE 18

Fusion of JUN amphipathic helix to the carboxy terminus of HBcAg(1-144)

[0331] The JUN helix was fused to the carboxy terminus of the HBcAg amino acid sequence 1 to 144 (HBcAg-JUN construct). For construction of the HBcAg-JUN DNA sequence, the sequences encoding the JUN helix and HBcAg(1-144) were amplified separately by PCR. The JUN sequence was amplified from the pJuFo plasmid with primers SacII-JUN(s) and JUN-HindIII(as). SacII-JUN(s) introduced a linker encoding amino acids LAAG. This sequence also contains a SacII site. JUN-HindIII(as) introduced a stop codon (TAA) followed by a HindIII site. The HBcAg(1-144) DNA sequence was amplified from the pEco63 plasmid using primers EcoRI-HBcAg(s) and HBcAg(1-144)-JUN(as). EcoRI-HBcAg(s) introduced an EcoRI site prior to the Start ATG of the HBcAg coding

sequence. HBcAg(1-144)-JUN(as) introduces a sequence encoding the peptide linker (LAAG), which also contains a SacII site. For the PCR reactions, 100 pmol of each oligo and 50 ng of the template DNAs were used in the 50 µl reaction mixtures with 2 units of Pwo polymerase, 0.1 mM dNTPs and 2 mM MgSO<sub>4</sub>. Temperature cycling was carried out as follows: 94°C for 2 minutes; and 30 cycles of 94°C (1 minute), 50°C (1 minute), 72°C (2 minutes).

**[0332]** Primer sequences

SacII-JUN(s):

(5'-CTAGCCGCGGGTTGCGGTGGTCGGATCGCCCGG-3') (SEQ ID NO:65);

JUN-HindIII(as):

(5'-CGCGTCCCAAGCTTTTAGCAACCAACGTGGTTCATGAC -3') (SEQ ID NO:66);

EcoRI-HBcAg(s):

(5'-CCGGAATTCATGGACATTGACCCTTATAAAG-3') (SEQ ID NO:67);  
and

HBcAg-JUN(as):

(5'-CCGACCACCGCAACCCGCGGCTAGCGGAAGCGTTGATAGGATAGG-3')  
(SEQ ID NO:68).

**[0333]** Fusion of the two PCR fragments was performed by PCR using primers EcoRI-HBcAg(s) and JUN-HindIII(as). For the PCR fusion, 100 pmol of each oligo was used with 100ng of the purified PCR fragments in a 50 µl reaction mixture containing 2 units of Pwo polymerase, 0.1 mM dNTPs and 2 mM MgSO<sub>4</sub>. PCR cycling conditions were: 94°C for 2 minutes; and 35 cycles of 94°C (1 minute), 50°C (1 minute), 72°C (2 minutes). The final PCR product was

analyzed by agarose gel electrophoresis, and digested for 16 hours in an appropriate buffer with EcoRI and HindIII restriction enzymes. The DNA fragment was gel purified and ligated into EcoRI/HindIII-digested pKK vector to generate pKK-HBcAg-JUN expression vector. Insertion of the PCR product was analyzed by EcoRI/HindIII restriction analysis and by DNA sequencing of the insert.

#### EXAMPLE 19

Insertion of JUN amphipathic helix into the c/e1 epitope of HBcAg(1-144)

[0334] The c/e1 epitope (residues 72 to 88) of HBcAg is known to be located in the tip region on the surface of the Hepatitis B virus capsid. A part of this region (residues 76 to 82) of the protein was genetically replaced by the JUN helix to provide an attachment site for antigens (HBcAg-JUNIns construct). The HBcAg-JUNIns DNA sequence was generated by PCRs: The JUN helix sequence and two sequences encoding HBcAg fragments (amino acid residues 1 to 75 and 83 to 144) were amplified separately by PCR. The JUN sequence was amplified from the pJuFo plasmid with primers BamHI-JUN(s) and JUN-SacII(as). BamHI-JUN(s) introduced a linker sequence encoding the peptide sequence GSGGG that also contains a BamHI site. JUN-SacII(as) introduced a sequence encoding the peptide linker GAAGS followed by a sequence complementary to the 3' end of the JUN coding sequence. The HBcAg(1-75) DNA sequence was amplified from the pEco63 plasmid using primers EcoRIHBcAg(s) and HBcAg75-JUN(as). EcoRIHBcAg(s) introduced an EcoRI site followed by a sequence corresponding to the 5' end of the HBcAg sequence. HBcAg75-JUN(as) introduced a linker encoding the peptide GSGGG after amino acid 75 of HBcAg followed by a sequence complementary to the 5' end of the sequence encoding the JUN helix. The HBcAg (83-144) fragment was amplified using primers JUN-HBcAg83(s) and HBcAg(1-144)Hind(as). JUN-HBcAg83(s) contained a sequence corresponding to the 3' end of the JUN-encoding sequence followed by a linker encoding the peptide, GAAGS and a sequence corresponding to the 5' end of the

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sequence encoding HBcAg (83-144). HBcAg(1-144)Hind(as) introduced a stop codon and a HindIII site after codon 144 of the HBcAg gene. For the PCR reactions, 100 pmol of each oligo and 50 ng of the template DNAs were used in the 50 µl reaction mixtures (2 units of Pwo polymerase, 0.1 mM dNTPs and 2 mM MgSO<sub>4</sub>). Temperature cycling was performed as follows: 94°C for 2 minutes; and 35 cycles of 94°C (1 minute), 50°C (1 minute), 72°C (2 minutes).

[0335] Primer sequences:

BamHI-JUN(s):

(5'-CTAATGGATCCGGTGGGGGCTGCGGTGGTCGGATCGCCCGGCTCGAG-3')  
(SEQ ID NO:69);

JUN-SacII(as):

(5'-GTCGCTACCCGCGGCTCCGCAACCAACGTGGTTCATGAC-3')(SEQ  
ID NO:70);

EcoRIHBcAg(s):

(5'- CCGGAATTCATGGACATTGACCCTTATAAAG-3') (SEQ ID NO:71);

HBcAg75-JUN (as):

(5'-CCGACCACCGCAGCCCCACCGGATCCATTAGTACCCACCCAGGTAGC-3')  
(SEQ ID NO:72);

JUN-HBcAg83(s):

(5'-GTTGGTTGCGGAGCCGCGGGTAGCGACCTAGTAGTCAGTTATGTC-3')  
(SEQ ID NO:73); and



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HBcAg(1-144)Hind(as):

(5'-CGCGTCCCAAGCTTCTACGGAAGCGTTGATAGGATAGG-3') (SEQ ID NO:74).

[0336] Fusion of the three PCR fragments was performed as follows. First, the fragment encoding HBcAg 1-75 was fused with the sequence encoding JUN by PCR using primers EcoRIHBcAg(s) and JUN-SacII(as). Second, the product obtained was fused with the HBcAg(83-144) fragment by PCR using primers EcoRI HBcAg(s) and HBcAg HindIII(as). For PCR fusions, 100 pmol of each oligo was used with 100 ng of the purified PCR fragments in a 50 µl reaction mixture containing 2 units of Pwo polymerase, 0.1 mM dNTPs and 2 mM MgSO<sub>4</sub>. The same PCR cycles were used as for generation of the individual fragments. The final PCR product was digested for 16 hours in an appropriate buffer with EcoRI and HindIII restriction enzymes. The DNA fragment was ligated into EcoRI/HindIII-digested pKK vector, yielding the pKK-HBcAg-JUNIns vector. Insertion of the PCR product was analyzed by EcoRI/HindIII restriction analysis and by DNA sequencing of the insert.

#### EXAMPLE 20

Fusion of the JUN amphipathic helix to the carboxy terminus of the measles virus nucleocapsid (N) protein

[0337] The JUN helix was fused to the carboxy terminus of the truncated measles virus N protein fragment comprising amino acid residues 1 to 473 (N473-JUN construct). For construction of the DNA sequence encoding N473-JUN the sequence encoding the JUN helix and the sequence encoding N473-JUN were amplified separately by PCR. The JUN sequence was amplified from the pJuFo plasmid with primers SacII-JUN(s) and JUN-HindIII(as). SacII-JUN(s) introduced a sequence encoding peptide linker LAAG. This sequence also contained a SacII site. The JUN-HindIII(as) anti-sense primer introduced a stop codon (TAA) followed by a HindIII site. The N (1-473) sequence was amplified

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from the pSC-N plasmid containing the complete measles virus N protein coding sequence (obtained from M. Billeter, Zurich) using primers EcoRI-Nmea(s) and Nmea-JUN(as). EcoRI-N(meas)(s) introduced an EcoRI site prior to the Start ATG of the N coding sequence. N(meas)-JUN(as) was complementary to the 3' end of the N(1-473) coding sequence followed by a sequence complementary to the coding sequence for the peptide linker (LAAG). For the PCR reactions, 100 pmol of each oligo and 50 ng of the template DNAs were used in the 50 µl reaction mixtures with 2 units of Pwo polymerase, 0.1 mM dNTPs and 2 mM MgSO<sub>4</sub>. Temperature cycling was performed as follows: 94°C for 2 minutes; and 35 cycles of 94°C (1 minute), 55°C (1 minute), 72°C (2 minutes).

[0338] Primer sequences:

SacII-JUN(s):

(5'-CTAGCCGCGGGTTGCGGTGGTCGGATCGCCCGG-3') (SEQ ID NO:75);

JUN-HindIII(as):

(5'-CGCGTCCCAAGCTTTTAGCAACCAACGTGGTTCATGAC -3') (SEQ ID NO:76);

EcoRI-Nmea(s):

(5'-CCGGAATTCATGGCCACACTTTTAAGGAGC-3') (SEQ ID NO:77); and

Nmea-JUN(as):

(5'-CGCGTCCCAAGCTTTTAGCAACCAACGTGGTTCATGAC-3') (SEQ ID NO:78).

Fusion of the two PCR fragments was performed in a further PCR using primers EcoRI-Nmea(s) and Nmea-JUN(as). For the PCR fusion, 100 pmol of

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each oligo was used with 100 ng of the purified PCR fragments in a 50  $\mu$ l reaction mixture containing 2 units of Pwo polymerase, 0.1 mM dNTPs and 2 mM  $MgSO_4$ . Temperature cycling was performed as follows: 94°C for 2 minutes; and 35 cycles of 94°C (1 minute), 50°C (1 minute), 72°C (2 minutes). The PCR product was digested for 16 hours in an appropriate buffer with EcoRI and HindIII restriction enzymes. The DNA fragment was gel purified and ligated into EcoRI/HindIII-digested pKK vector, yielding the pKK-N473-JUN plasmid. Insertion of the PCR product was analyzed by EcoRI/HindIII restriction analysis and by DNA sequencing of the insert.

#### Example 21

##### Expression and partial purification of HBcAg-JUN

[0339] *E. coli* strain XL-1 blue was transformed with pKK-HBcAg-JUN. 1 ml of an overnight culture of bacteria was used to inoculate 100 ml of LB medium containing 100  $\mu$ g/ml ampicillin. This culture was grown for 4 hours at 37°C until an OD at 600 nm of approximately 0.8 was reached. Induction of the synthesis of HBcAg-JUN was performed by addition of IPTG to a final concentration of 1 mM. After induction, bacteria were further shaken at 37°C for 16 hours. Bacteria were harvested by centrifugation at 5000 x g for 15 minutes. The pellet was frozen at -20°C. The pellet was thawed and resuspended in bacteria lysis buffer (10 mM  $Na_2HPO_4$ , pH 7.0, 30 mM NaCl, 0.25% Tween-20, 10 mM EDTA, 10 mM DTT) supplemented with 200  $\mu$ g/ml lysozyme and 10  $\mu$ l of Benzonase (Merck). Cells were incubated for 30 minutes at room temperature and disrupted using a French pressure cell. Triton X-100 was added to the lysate to a final concentration of 0.2%, and the lysate was incubated for 30 minutes on ice and shaken occasionally. Figure 4 shows HBcAg-JUN protein expression in *E. coli* upon induction with IPTG. *E. coli* cells harboring pKK-HBcAg-JUN expression plasmid or a control plasmid were used for induction of HBcAg-JUN expression with IPTG. Prior to the addition of IPTG, a sample was removed from the bacteria culture carrying the pKK-HBcAg-JUN plasmid (lane 3) and from a

culture carrying the control plasmid (lane 1). Sixteen hours after addition of IPTG, samples were again removed from the culture containing pKK-HBcAg-JUN (lane 4) and from the control culture (lane 2). Protein expression was monitored by SDS-PAGE followed by Coomassie staining.

[0340] The lysate was then centrifuged for 30 minutes at 12,000 x g in order to remove insoluble cell debris. The supernatant and the pellet were analyzed by Western blotting using a monoclonal antibody against HBcAg (YVS1841, purchased from Accurate Chemical and Scientific Corp., Westbury, NY, USA), indicating that a significant amount of HBcAg-JUN protein was soluble (Fig. 5). Briefly, lysates from *E. coli* cells expressing HBcAg-JUN and from control cells were centrifuged at 14,000 x g for 30 minutes. Supernatant (= soluble fraction) and pellet (= insoluble fraction) were separated and diluted with SDS sample buffer to equal volumes. Samples were analyzed by SDS-PAGE followed by Western blotting with anti-HBcAg monoclonal antibody YVS 1841. Lane 1: soluble fraction, control cells; lane 2: insoluble fraction, control cells; lane 3: soluble fraction, cells expressing HBcAg-JUN; lane 4: insoluble fraction, cells expressing HBcAg-JUN.

[0341] The cleared cell lysate was used for step-gradient centrifugation using a sucrose step gradient consisting of a 4 ml 65% sucrose solution overlaid with 3 ml 15% sucrose solution followed by 4 ml of bacterial lysate. The sample was centrifuged for 3 hrs with 100,000 x g at 4°C. After centrifugation, 1 ml fractions from the top of the gradient were collected and analyzed by SDS-PAGE followed by Coomassie staining. (Fig. 6). Lane 1: total *E. coli* lysate prior to centrifugation. Lane 1 and 2: fractions 1 and 2 from the top of the gradient. Lane 4 to 7: fractions 5 to 8 (15% sucrose). The HBcAg-JUN protein was detected by Coomassie staining.

[0342] The HBcAg-JUN protein was enriched at the interface between 15 and 65% sucrose indicating that it had formed a capsid particle. Most of the bacterial proteins remained in the sucrose-free upper layer of the gradient, therefore step-

gradient centrifugation of the HBcAg-JUN particles led both to enrichment and to a partial purification of the particles.

## EXAMPLE 22

### Covalent Coupling of hGH-FOS to HBcAg-JUN

[0343] In order to demonstrate binding of a protein to HBcAg-JUN particles, we chose human growth hormone (hGH) fused with its carboxy terminus to the FOS helix as a model protein (hGH-FOS). HBcAg-JUN particles were mixed with partially purified hGH-FOS and incubated for 4 hours at 4 °C to allow binding of the proteins. The mixture was then dialyzed overnight against a 3000-fold volume of dialysis buffer (150 mM NaCl, 10 mM Tris-HCl solution, pH 8.0) in order to remove DTT present in both the HBcAg-JUN solution and the hGH-FOS solution and thereby allow covalent coupling of the proteins through the establishment of disulfide bonds. As controls, the HBcAg-JUN and the hGH-FOS solutions were also dialyzed against dialysis buffer. Samples from all three dialyzed protein solutions were analyzed by SDS-PAGE under non-reducing conditions. Coupling of hGH-FOS to HBcAg-JUN was detected in an anti-hGH immunoblot (Fig. 7). hGH-FOS bound to HBcAg-JUN should migrate with an apparent molecular mass of approximately 53 kDa, while unbound hGH-FOS migrates with an apparent molecular mass of 31 kDa. The dialysate was analyzed by SDS-PAGE in the absence of reducing agent (lane 3) and in the presence of reducing agent (lane 2) and detected by Coomassie staining. As a control, hGH-FOS that had not been mixed with capsid particles was also loaded on the gel in the presence of reducing agent (lane 1).

[0344] A shift of hGH-FOS to a molecular mass of approximately 53 kDa was observed in the presence of HBcAg-JUN capsid protein, suggesting that efficient binding of hGH-FOS to HBcAg-JUN had taken place.

## EXAMPLE 23

Insertion of a peptide containing a Lysine residue into the  
c/e1 epitope of HBcAg(1-149)

- [0345] The c/e1 epitope (residues 72 to 88) of HBcAg is located in the tip region on the surface of the Hepatitis B virus capsid (HBcAg). A part of this region (Proline 79 and Alanine 80) was genetically replaced by the peptide Gly-Gly-Lys-Gly-Gly (HBcAg-Lys construct). The introduced Lysine residue contains a reactive amino group in its side chain that can be used for intermolecular chemical crosslinking of HBcAg particles with any antigen containing a free cysteine group.
- [0346] HBcAg-Lys DNA, having the amino acid sequence shown in SEQ ID NO:158, was generated by PCRs. The two fragments encoding HBcAg fragments (amino acid residues 1 to 78 and 81 to 149) were amplified separately by PCR. The primers used for these PCRs also introduced a DNA sequence encoding the Gly-Gly-Lys-Gly-Gly peptide. The HBcAg (1 to 78) fragment was amplified from pEco63 using primers EcoRIHBcAg(s) and Lys-HBcAg(as). The HBcAg (81 to 149) fragment was amplified from pEco63 using primers Lys-HBcAg(s) and HBcAg(1-149)Hind(as). Primers Lys-HBcAg(as) and Lys-HBcAg(s) introduced complementary DNA sequences at the ends of the two PCR products allowing fusion of the two PCR products in a subsequent assembly PCR. The assembled fragments were amplified by PCR using primers EcoRIHBcAg(s) and HbcAg(1-149)Hind(as).
- [0347] For the PCRs, 100 pmol of each oligo and 50 ng of the template DNAs were used in the 50  $\mu$ l reaction mixtures with 2 units of Pwo polymerase, 0.1 mM dNTPs and 2 mM MgSO<sub>4</sub>. For both reactions, temperature cycling was carried out as follows: 94°C for 2 minutes; 30 cycles of 94°C (1 minute), 50°C (1 minute), 72°C (2 minutes).

**[0348]** Primer sequences:

EcoRIHBcAg(s):

(5'-CCGGAATTCATGGACATTGACCCTTATAAAG-3') (SEQ ID NO:79);

Lys-HBcAg(as):

(5'-CCTAGAGCCACCTTTGCCACCATCTTCTAAATTAGTACCCACCCAG  
GTAGC-3') (SEQ ID NO:80);

Lys-HBcAg(s):

(5'-GAAGATGGTGGCAAAGGTGGCTCTAGGGACCTAGTAGTCAGTTAT  
GTC -3') (SEQ ID NO:81);

HBcAg(1-149)Hind(as):

(5'-CGCGTCCCAAGCTTCTAAACAACAGTAGTCTCCGGAAG-3') (SEQ ID  
NO:82).

**[0349]** For fusion of the two PCR fragments by PCR 100 pmol of primers EcoRIHBcAg(s) and HBcAg(1-149)Hind(as) were used with 100 ng of the two purified PCR fragments in a 50 µl reaction mixture containing 2 units of Pwo polymerase, 0.1 mM dNTPs and 2 mM MgSO<sub>4</sub>. PCR cycling conditions were: 94°C for 2 minutes; 30 cycles of 94°C (1 minute), 50°C (1 minute), 72°C (2 minutes). The assembled PCR product was analyzed by agarose gel electrophoresis, purified and digested for 19 hours in an appropriate buffer with EcoRI and HindIII restriction enzymes. The digested DNA fragment was ligated into EcoRI/HindIII-digested pKK vector to generate pKK-HBcAg-Lys expression vector. Insertion of the PCR product into the vector was analyzed by EcoRI/HindIII restriction analysis and DNA sequencing of the insert.

**[0350]** The amino acid sequence of the HBcAg-Lys polypeptide is  
MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYREAIESPEHCSP

HHTALRQAILCWGELMTLATWVGNTLEDGGKGGSRDLVVSIVNTNM  
GLKIRQLLWFHISCLTFGRETIVLEYLVSFQVWIRTPPAYRPPNAPILSTL  
PETTVV (SEQ ID NO: 185). This sequence differs from SEQ ID NO:134 at  
amino acid 74 (N in SEQ ID NO:134, T in SEQ ID NO:185) and at amino acid  
87 (N in SEQ ID NO:134, S in SEQ ID NO: 185).

#### EXAMPLE 24

##### Expression and partial purification of HBcAg-Lys

[0351] *E. coli* strain XL-1 blue was transformed with pKK-HBcAg-Lys. 1 ml of an overnight culture of bacteria was used to inoculate 100 ml of LB medium containing 100 µg/ml ampicillin. This culture was grown for 4 hours at 37°C until an OD at 600 nm of approximately 0.8 was reached. Induction of the synthesis of HBcAg-Lys was performed by addition of IPTG to a final concentration of 1 mM. After induction, bacteria were further shaken at 37°C for 16 hours. Bacteria were harvested by centrifugation at 5000 x g for 15 minutes. The pellet was frozen at -20°C. The pellet was thawed and resuspended in bacteria lysis buffer (10 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.0, 30 mM NaCl, 0.25% Tween-20, 10 mM EDTA, 10 mM DTT) supplemented with 200 µg/ml lysozyme and 10 µl of Benzonase (Merck). Cells were incubated for 30 minutes at room temperature and disrupted using a French pressure cell. Triton X-100 was added to the lysate to a final concentration of 0.2%, and the lysate was incubated for 30 minutes on ice and shaken occasionally. *E. coli* cells harboring pKK-HBcAg-Lys expression plasmid or a control plasmid were used for induction of HBcAg-Lys expression with IPTG. Prior to the addition of IPTG, a sample was removed from the bacteria culture carrying the pKK-HBcAg-Lys plasmid and from a culture carrying the control plasmid. Sixteen hours after addition of IPTG, samples were again removed from the culture containing pKK-HBcAg-Lys and from the control culture. Protein expression was monitored by SDS-PAGE followed by Coomassie staining.



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- [0352] The lysate was then centrifuged for 30 minutes at 12,000 x g in order to remove insoluble cell debris. The supernatant and the pellet were analyzed by Western blotting using a monoclonal antibody against HBcAg (YVS1841, purchased from Accurate Chemical and Scientific Corp., Westbury, NY, USA), indicating that a significant amount of HBcAg-Lys protein was soluble. Briefly, lysates from *E. coli* cells expressing HBcAg-Lys and from control cells were centrifuged at 14,000 x g for 30 minutes. Supernatant (= soluble fraction) and pellet (= insoluble fraction) were separated and diluted with SDS sample buffer to equal volumes. Samples were analyzed by SDS-PAGE followed by Western blotting with anti-HBcAg monoclonal antibody YVS 1841.
- [0353] The cleared cell lysate was used for step-gradient centrifugation using a sucrose step gradient consisting of a 4 ml 65% sucrose solution overlaid with 3 ml 15% sucrose solution followed by 4 ml of bacterial lysate. The sample was centrifuged for 3 hrs with 100,000 x g at 4 °C. After centrifugation, 1 ml fractions from the top of the gradient were collected and analyzed by SDS-PAGE followed by Coomassie staining. The HBcAg-Lys protein was detected by Coomassie staining.
- [0354] The HBcAg-Lys protein was enriched at the interface between 15 and 65% sucrose indicating that it had formed a capsid particle. Most of the bacterial proteins remained in the sucrose-free upper layer of the gradient, therefore step-gradient centrifugation of the HBcAg-Lys particles led both to enrichment and to a partial purification of the particles.

#### EXAMPLE 25

Chemical coupling of FLAG peptide to HBcAg-Lys  
using the heterobifunctional cross-linker SPDP

- [0355] Synthetic FLAG peptide with a Cysteine residue at its amino terminus (amino acid sequence CGGDYKDDDDK (SEQ ID NO:147)) was coupled chemically to purified HBcAg-Lys particles in order to elicit an immune response against the FLAG peptide. 600 µl of a 95% pure solution of HBcAg-Lys particles

(2 mg/ml) were incubated for 30 minutes at room temperature with the heterobifunctional cross-linker N-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP) (0.5 mM). After completion of the reaction, the mixture was dialyzed overnight against 1 liter of 50 mM Phosphate buffer (pH 7.2) with 150 mM NaCl to remove free SPDP. Then 500 µl of derivatized HBcAg-Lys capsid (2 mg/ml) were mixed with 0.1 mM FLAG peptide (containing an amino-terminal cysteine) in the presence of 10 mM EDTA to prevent metal-catalyzed sulphydryl oxidation. The reaction was monitored through the increase of the optical density of the solution at 343 nm due to the release of pyridine-2-thione from SPDP upon reaction with the free cysteine of the peptide. The reaction of derivatized Lys residues with the peptide was complete after approximately 30 minutes.

[0356] The FLAG decorated particles were injected into mice.

#### EXAMPLE 26

##### Construction of pMPSV-gp140cys

[0357] The gp140 gene was amplified by PCR from pCytTSgp140FOS using oligos gp140CysEcoRI and SalIgp140. For the PCRs, 100 pmol of each oligo and 50 ng of the template DNAs were used in the 50 µl reaction mixtures with 2 units of Pwo polymerase, 0.1 mM dNTPs and 2 mM MgSO<sub>4</sub>. For both reactions, temperature cycling was carried out as follows: 94°C for 2 minutes; 30 cycles of 94°C (0.5 minutes), 55°C (0.5 minutes), 72°C (2 minutes).

[0358] The PCR product was purified using QiaEXII kit, digested with SalI/EcoRI and ligated into vector pMPSVHE cleaved with the same enzymes.

[0359] Oligo sequences:

Gp140CysEcoRI:

5'-GCCGAATTCCTAGCAGCTAGCACCGAATTTATCTAA-3' (SEQ ID NO:83);

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SalIgp140:

5'-GGTTAAGTCGACATGAGAGTGAAGGAGAAATAT-3' (SEQ ID NO:84).

## EXAMPLE 27

## Expression of pMPSVgp140Cys

[0360] pMPSVgp140Cys (20 µg) was linearized by restriction digestion. The reaction was stopped by phenol/chloroform extraction, followed by an isopropanol precipitation of the linearized DNA. The restriction digestion was evaluated by agarose gel electrophoresis. For the transfection, 5.4 µg of linearized pMPSVgp140-Cys was mixed with 0.6 µg of linearized pSV2Neo in 30 µl H<sub>2</sub>O and 30 µl of 1 M CaCl<sub>2</sub> solution was added. After addition of 60 µl phosphate buffer (50 mM HEPES, 280 mM NaCl, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.05), the solution was vortexed for 5 seconds, followed by an incubation at room temperature for 25 seconds. The solution was immediately added to 2 ml HP-1 medium containing 2% FCS (2% FCS medium). The medium of an 80% confluent BHK21 cell culture (6-well plate) was then replaced by the DNA containing medium. After an incubation for 5 hours at 37°C in a CO<sub>2</sub> incubator, the DNA containing medium was removed and replaced by 2 ml of 15% glycerol in 2% FCS medium. The glycerol containing medium was removed after a 30 second incubation phase, and the cells were washed by rinsing with 5 ml of HP-1 medium containing 10% FCS. Finally 2 ml of fresh HP-1 medium containing 10% FCS was added.

[0361] Stably transfected cells were selected and grown in selection medium (HP-1 medium supplemented with G418) at 37°C in a CO<sub>2</sub> incubator. When the mixed population was grown to confluency, the culture was split to two dishes, followed by a 12 h growth period at 37°C. One dish of the cells was shifted to 30°C to induce the expression of soluble GP140-FOS. The other dish was kept at 37°C.

[0362] The expression of soluble GP140-Cys was determined by Western blot analysis. Culture media (0.5 ml) was methanol/chloroform precipitated, and the

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pellet was resuspended in SDS-PAGE sample buffer. Samples were heated for 5 minutes at 95°C before being applied to a 15% acrylamide gel. After SDS-PAGE, proteins were transferred to Protan nitrocellulose membranes (Schleicher & Schuell, Germany) as described by Bass and Yang, in Creighton, T.E., ed., *Protein Function: A Practical Approach*, 2nd Edn., IRL Press, Oxford (1997), pp. 29-55. The membrane was blocked with 1 % bovine albumin (Sigma) in TBS (10xTBS per liter: 87.7 g NaCl, 66.1g Trizma hydrochloride (Sigma) and 9.7 g Trizma base (Sigma), pH 7.4) for 1 hour at room temperature, followed by an incubation with an anti-GP140 or GP-160 antibody for 1 hour. The blot was washed 3 times for 10 minutes with TBS-T (TBS with 0.05% Tween20), and incubated for 1 hour with an alkaline-phosphatase-anti-mouse/rabbit/monkey/human IgG conjugate. After washing 2 times for 10 minutes with TBS-T and 2 times for 10 minutes with TBS, the development reaction was carried out using alkaline phosphatase detection reagents (10 ml AP buffer (100 mM Tris/HCl, 100 mM NaCl, pH 9.5) with 50 µl NBT solution (7.7% Nitro Blue Tetrazolium (Sigma) in 70% dimethylformamide) and 37 µl of X-Phosphate solution (5% of 5-bromo-4-chloro-3-indolyl phosphate in dimethylformamide)).

## EXAMPLE 28

### Purification of gp140Cys

- [0363] An anti-gp120 antibody was covalently coupled to a NHS/EDC activated dextran and packed into a chromatography column. The supernatant, containing GP140Cys is loaded onto the column and after sufficient washing, GP140Cys was eluted using 0.1 M HCl. The eluate was directly neutralized during collection using 1 M Tris pH 7.2 in the collection tubes.
- [0364] Disulfide bond formation might occur during purification, therefore the collected sample is treated with 10 mM DTT in 10 mM Tris pH 7.5 for 2 hours at 25°C.

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- [0365] DTT is removed by subsequent dialysis against 10 mM Mes; 80 mM NaCl pH 6.0. Finally GP140Cys is mixed with alphavirus particles containing the *JUN* residue in E2 as described in Example 16.

## EXAMPLE 29

## Construction of PLA2-Cys

- [0366] The PLA2 gene was amplified by PCR from pAV3PLAfos using oligos EcoRIPLA and PLA-Cys-hind. For the PCRs, 100 pmol of each oligo and 50 ng of the template DNAs were used in the 50  $\mu$ l reaction mixtures with 2 units of Pwo polymerase, 0.1 mM dNTPs and 2 mM MgSO<sub>4</sub>. For both reactions, temperature cycling was carried out as follows: 94°C for 2 minutes; 30 cycles of 94°C (0.5 minutes), 55°C (0.5 minutes), 72°C (2 minutes).
- [0367] The PCR product was purified using QiaEXII kit, digested with EcoRI/HinDIII and ligated into vector pAV3 cleaved with the same enzymes.

- [0368] Oligos

EcoRIPLA:

5'-TAACCGAATTCAGGAGGTAAAAAGATATGG-3' (SEQ ID NO:85)

PLACys-hind:

5'-GAAGTAAAGCTTTTAACCACCGCAACCACCAGAAG-3' (SEQ ID NO:86).

## EXAMPLE 30

## Expression and Purification of PLA-Cys

- [0369] For cytoplasmic production of Cys tagged proteins, *E. coli* XL-1-Blue strain was transformed with the vectors pAV3::PLA and pPLA-Cys. The culture was incubated in rich medium in the presence of ampicillin at 37°C with shaking. At an optical density (550nm) of , 1 mM IPTG was added and incubation was continued for another 5 hours. The cells were harvested by centrifugation,

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resuspended in an appropriate buffer (e.g., Tris-HCl, pH 7.2, 150 mM NaCl) containing DNase, RNase and lysozyme, and disrupted by passage through a french pressure cell. After centrifugation (Sorvall RC-5C, SS34 rotor, 15000 rpm, 10 min, 4°C), the pellet was resuspended in 25 ml inclusion body wash buffer (20 mM tris-HCl, 23% sucrose, 0.5% Triton X-100, 1 mM EDTA, pH8) at 4°C and recentrifuged as described above. This procedure was repeated until the supernatant after centrifugation was essentially clear. Inclusion bodies were resuspended in 20 ml solubilization buffer (5.5 M guanidinium hydrochloride, 25 mM tris-HCl, pH 7.5) at room temperature and insoluble material was removed by centrifugation and subsequent passage of the supernatant through a sterile filter (0.45 µm). The protein solution was kept at 4°C for at least 10 hours in the presence of 10 mM EDTA and 100 mM DTT and then dialyzed three times against 10 volumes of 5.5 M guanidinium hydrochloride, 25 mM tris-HCl, 10 mM EDTA, pH 6. The solution was dialyzed twice against 5 l 2 M urea, 4 mM EDTA, 0.1 M NH<sub>4</sub>Cl, 20 mM sodium borate (pH 8.3) in the presence of an appropriate redox shuffle (oxidized glutathione/reduced glutathione; cystine/cysteine). The refolded protein was then applied to an ion exchange chromatography. The protein was stored in an appropriate buffer with a pH above 7 in the presence of 2-10 mM DTT to keep the cysteine residues in a reduced form. Prior to coupling of the protein with the alphavirus particles, DTT was removed by passage of the protein solution through a Sephadex G-25 gel filtration column.

### EXAMPLE 31

Construction of a HBcAg devoid of free cysteine residues and containing an inserted lysine residue

[0370] A Hepatitis core Antigen (HBcAg), referred to herein as HBcAg-lys-2cys-Mut, devoid of cysteine residues at positions corresponding to 48 and 107 in SEQ ID NO:134 and containing an inserted lysine residue was constructed using the following methods.

[0371] The two mutations were introduced by first separately amplifying three fragments of the HBcAg-Lys gene prepared as described above in Example 23 with the following PCR primer combinations. PCR methods essentially as described in Example 1 and conventional cloning techniques were used to prepare the HBcAg-lys-2cys-Mut gene.

[0372] In brief, the following primers were used to prepare fragment 1:

Primer 1: EcoRIHBcAg(s)

CCGGAATTCATGGACATTGACCCTTATAAAG (SEQ ID NO:148)

Primer 2: 48as

GTGCAGTATGGTGAGGTGAGGAATGCTCAGGAGACTC (SEQ ID NO:149)

[0373] The following primers were used to prepare fragment 2:

Primer 3: 48s

GSGTCTCCTGAGCATTCTCACCTCACCATACTGCAC (SEQ ID NO:150)

Primer 4: 107as

CTTCCAAAAGTGAGGGAAGAAATGTGAAACCAC (SEQ ID NO:151)

[0374] The following primers were used to prepare fragment 3:

Primer 5: HBcAg149hind-as

CGCGTCCCAAGCTTCTAAACAACAGTAGTCTCCGGAAGCGTTGATAG  
(SEQ ID NO:152)

Primer 6: 107s

GTGGTTTCACATTTCTTCCCTCACTTTTGGAAG (SEQ ID NO:153)

[0375] Fragments 1 and 2 were then combined with PCR primers EcoRIHBcAg(s) and 107as to give fragment 4. Fragment 4 and fragment 3 were

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then combined with primers EcoRIHBcAg(s) and HBcAg149hind-as to produce the full length gene. The full length gene was then digested with the EcoRI (GAATTC) and HindIII (AAGCTT) enzymes and cloned into the pKK vector (Pharmacia) cut at the same restriction sites. The amino acid sequence of the HBcAg-Lys-2cys-Mut polypeptide is MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYREALESPEHSSPHHTALRQAILCWGELMTLATWVGTNLEDGGKGGSRDLVVS YVNTNMGLKIRQLLWFHISSLTFGR ETVLEYLVSFVWIRTTPPAYRPPNAPILSTLPETTVV (SEQ ID NO: 186).

### EXAMPLE 32

Blockage of free cysteine residues of a HBcAg followed by cross-linking

[0376] The free cysteine residues of the HBcAg-Lys prepared as described above in Example 23 were blocked using Iodacetamide. The blocked HBcAg-Lys was then cross-linked to the FLAG peptide with the hetero-bifunctional cross-linker m-maleimidonbenzoyl-N-hydroxysuccinimide ester (Sulfo-MBS).

[0377] The methods used to block the free cysteine residues and cross-link the HBcAg-Lys are as follows. HBcAg-Lys (550 µg/ml) was reacted for 15 minutes at room temperature with Iodacetamide (Fluka Chemie, Brugg, Switzerland) at a concentration of 50 mM in phosphate buffered saline (PBS) (50 mM sodium phosphate, 150 mM sodium chloride), pH 7.2, in a total volume of 1 ml. The so modified HBcAg-Lys was then reacted immediately with Sulfo-MBS (Pierce) at a concentration of 530 µM directly in the reaction mixture of step 1 for 1 hour at room temperature. The reaction mixture was then cooled on ice, and dialyzed against 1000 volumes of PBS pH 7.2. The dialyzed reaction mixture was finally reacted with 300 µM of the FLAG peptide (CGGDYKDDDDK (SEQ ID NO:147)) containing an N-terminal free cysteine for coupling to the activated HBcAg-Lys, and loaded on SDS-PAGE for analysis.

[0378] As shown in Figure 8, the resulting patterns of bands on the SDS-PAGE gel showed a clear additional band migrating slower than the control HBcAg-Lys derivatized with the cross-linker, but not reacted with the FLAG peptide.



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Reactions done under the same conditions without prior derivatization of the cysteines with Iodacetamide led to complete cross-linking of monomers of the HBcAg-Lys to higher molecular weight species.

### EXAMPLE 33

Isolation of Type-1 pili and chemical coupling of FLAG peptide to Type-1 pili of *Escherichia coli* using a heterobifunctional cross-linker

#### A. Introduction

[0379] Bacterial pili or fimbriae are filamentous surface organelles produced by a wide range of bacteria. These organelles mediate the attachment of bacteria to surface receptors of host cells and are required for the establishment of many bacterial infections like cystitis, pyelonephritis, new born meningitis and diarrhea.

[0380] Pili can be divided in different classes with respect to their receptor specificity (agglutination of blood cells from different species), their assembly pathway (extracellular nucleation, general secretion, chaperone/usher, alternate chaperone) and their morphological properties (thick, rigid pili; thin, flexible pili; atypical structures including capsule; curli; etc). Examples of thick, rigid pili forming a right handed helix that are assembled via the so called chaperone/usher pathway and mediate adhesion to host glycoproteins include Type-1 pili, P-pili, S-pili, F1C-pili, and 987P-pili). The most prominent and best characterized members of this class of pili are P-pili and Type-1 pili (for reviews on adhesive structures, their assembly and the associated diseases see Soto, G. E. & Hultgren, S. J., *J. Bacteriol.* 181:1059-1071 (1999); Bullitt & Makowski, *Biophys. J.* 74:623-632 (1998); Hung, D. L. & Hultgren, S. J., *J. Struct. Biol.* 124:201-220 (1998)).

[0381] Type-1 pili are long, filamentous polymeric protein structures on the surface of *E. coli*. They possess adhesive properties that allow for binding to mannose-containing receptors present on the surface of certain host tissues. Type-1 pili can be expressed by 70-80% of all *E. coli* isolates and a single *E. coli* cell can bear up to 500 pili. Type- pili reach a length of typically 0.2 to 2  $\mu\text{M}$  with

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an average number of 1000 protein subunits that associate to a right-handed helix with 3.125 subunits per turn with a diameter of 6 to 7 nm and a central hole of 2.0 to 2.5 nm.

- [0382] The main Type-1 pilus component, FimA, which represents 98% of the total pilus protein, is a 15.8 kDa protein. The minor pilus components FimF, FimG and FimH are incorporated at the tip and in regular distances along the pilus shaft (Klemm, P. & Krogfelt, K. A., "Type I fimbriae of *Escherichia coli*," in: *Fimbriae*. Klemm, P. (ed.), CRC Press Inc., (1994) pp. 9-26). FimH, a 29.1 kDa protein, was shown to be the mannose-binding adhesin of Type-1 pili (Krogfelt, K. A., *et al.*, *Infect. Immun.* 58:1995-1998 (1990); Klemm, P., *et al.*, *Mol. Microbiol.* 4:553-560 (1990); Hanson, M. S. & Brinton, C. C. J., *Nature* 17:265-268 (1988)), and its incorporation is probably facilitated by FimG and FimF (Klemm, P. & Christiansen, G., *Mol. Gen. Genetics* 208:439-445 (1987); Russell, P. W. & Orndorff, P. E., *J. Bacteriol.* 174:5923-5935 (1992)). Recently, it was shown that FimH might also form a thin tip-fibrillum at the end of the pili (Jones, C. H., *et al.*, *Proc. Nat. Acad. Sci. USA* 92:2081-2085 (1995)). The order of major and minor components in the individual mature pili is very similar, indicating a highly ordered assembly process (Soto, G. E. & Hultgren, S. J., *J. Bacteriol.* 181:1059-1071 (1999)).
- [0383] P-pili of *E. coli* are of very similar architecture, have a diameter of 6.8 nm, an axial hole of 1.5 nm and 3.28 subunits per turn (Bullitt & Makowski, *Biophys. J.* 74:623-632 (1998)). The 16.6 kDa PapA is the main component of this pilus type and shows 36% sequence identity and 59% similarity to FimA (see Table 1). As in Type-1 pili the 36.0 kDa P-pilus adhesin PapG and specialized adapter proteins make up only a tiny fraction of total pilus protein. The most obvious difference to Type-1 pili is the absence of the adhesin as an integral part of the pilus rod, and its exclusive localization in the tip fibrillum that is connected to the pilus rod via specialized adapter proteins that Type-1 pili lack (Hultgren, S. J., *et al.*, *Cell* 73:887-901 (1993)).

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- [0384] Table 1: Similarity and identity between several structural pilus proteins of Type-1 and P-pili (in percent). The adhesins were omitted.

		<u>Similarity</u>								
		FimA	PapA	FimI	FimF	FimG	PapE	PapK	PapH	PapF
<u>Identity</u>	FimA		59	57	56	44	50	44	46	46
	PapA	36		49	48	41	45	49	49	47
	FimI	35	31		56	46	40	47	48	48
	FimF	34	26	30		40	47	43	49	48
	FimG	28	28	28	26		39	39	41	45
	PapE	25	23	18	28	22		43	47	54
	PapK	24	29	25	28	22	18		49	53
	PapH	22	26	22	22	23	24	23		41
	PapF	18	22	22	24	28	27	26	21	

- [0385] Type-1 pili are extraordinary stable hetero-oligomeric complexes. Neither SDS-treatment nor protease digestions, boiling or addition of denaturing agents can dissociate Type-1 pili into their individual protein components. The combination of different methods like incubation at 100°C at pH 1.8 was initially found to allow for the depolymerization and separation of the components (Eshdat, Y., *et al.*, *J. Bacteriol.* 148:308-314 (1981); Brinton, C.C. J., *Trans. N. Y. Acad. Sci.* 27:1003-1054 (1965); Hanson, A. S., *et al.*, *J. Bacteriol.*, 170:3350-3358 (1988); Klemm, P. & Krogfelt, K. A., "Type I fimbriae of *Escherichia coli*," in: *Fimbriae*. Klemm, P. (ed.), CRC Press Inc., (1994) pp. 9-26). Interestingly, Type-1 pili show a tendency to break at positions where FimH is incorporated upon mechanical agitation, resulting in fragments that present a FimH adhesin at their tips. This was interpreted as a mechanism of the bacterium to shorten pili to an effective length under mechanical stress (Klemm, P. & Krogfelt, K. A., "Type I fimbriae of *Escherichia coli*," in: *Fimbriae*. Klemm, P. (ed.), CRC Press Inc., (1994) pp. 9-26). Despite their extraordinary stability, Type-1 pili have been shown to unravel partially in the presence of 50% glycerol; they lose their helical structure and form an extended and flexible, 2 nm wide protein chain (Abraham, S. N., *et al.*, *J. Bacteriol.* 174:5145-5148 (1992)).

- [0386] P-pili and Type-1 pili are encoded by single gene clusters on the *E. coli* chromosome of approximately 10 kb (Klemm, P. & Krogfelt, K. A., "Type I fimbriae of *Escherichia coli*," in: *Fimbriae*. Klemm, P. (ed.), CRC Press Inc., (1994) pp. 9-26; Orndorff, P. E. & Falkow, S., *J. Bacteriol.* 160:61-66 (1984)). A total of nine genes are found in the Type-1 pilus gene cluster, and 11 genes in the P-pilus cluster (Hultgren, S. J., *et al.*, *Adv. Prot. Chem.* 44:99-123 (1993)). Both clusters are organized quite similarly.
- [0387] The first two *fim*-genes, *fimB* and *fimE*, code for recombinases involved in the regulation of pilus expression (McClain, M. S., *et al.*, *J. Bacteriol.* 173:5308-5314 (1991)). The main structural pilus protein is encoded by the next gene of the cluster, *fimA* (Klemm, P., *Euro. J. Biochem.* 143:395-400 (1984); Orndorff, P. E. & Falkow, S., *J. Bacteriol.* 160:61-66 (1984); Orndorff, P. E. & Falkow, S., *J. Bacteriol.* 162:454-457 (1985)). The exact role of *fimI* is unclear. It has been reported to be incorporated in the pilus as well (Klemm, P. & Krogfelt, K. A., "Type I fimbriae of *Escherichia coli*," in: *Fimbriae*. Klemm, P. (ed.), CRC Press Inc., (1994) pp. 9-26). The adjacent *fimC* codes not for a structural component of the mature pilus, but for a so-called pilus chaperone that is essential for the pilus assembly (Klemm, P., *Res. Microbiol.* 143:831-838 (1992); Jones, C. H., *et al.*, *Proc. Nat. Acad. Sci. USA* 90:8397-8401 (1993)).
- [0388] The assembly platform in the outer bacterial membrane to which the mature pilus is anchored is encoded by *fimD* (Klemm, P. & Christiansen, G., *Mol. Gen. Genetics* 220:334-338 (1990)). The three minor components of the Type-1 pili, FimF, FimG and FimH are encoded by the last three genes of the cluster (Klemm, P. & Christiansen, G., *Mol. Gen. Genetics* 208:439-445 (1987)). Apart from *fimB* and *fimE*, all genes encode precursor proteins for secretion into the periplasm via the sec-pathway.
- [0389] The similarities between different pili following the chaperone/usher pathway are not restricted to their morphological properties. Their genes are also arranged in a very similar manner. Generally the gene for the main structural subunit is found directly downstream of the regulatory elements at the beginning

of the gene cluster, followed by a gene for an additional structural subunit (*fimI* in the case of Type-1 pili and *papH* in the case of P-pili). PapH was shown and FimI is supposed to terminate pilus assembly (Hultgren, S. J., *et al.*, *Cell* 73:887-901 (1993)). The two proteins that guide the process of pilus formation, namely the specialized pilus chaperone and the outer membrane assembly platform, are located adjacently downstream. At the end of the clusters a variable number of minor pilus components including the adhesins are encoded. The similarities in morphological structure, sequence (see Table 1), genetic organization and regulation indicate a close evolutionary relationship and a similar assembly process for these cell organelles.

[0390] Bacteria producing Type-1 pili show a so-called phase-variation. Either the bacteria are fully pilated or bald. This is achieved by an inversion of a 314 bp genomic DNA fragment containing the *fimA* promoter, thereby inducing an “all on” or “all off” expression of the pilus genes (McClain, M. S., *et al.*, *J. Bacteriol.* 173:5308-5314 (1991)). The coupling of the expression of the other structural pilus genes to *fimA* expression is achieved by a still unknown mechanism. However, a wide range of studies elucidated the mechanism that influences the switching between the two phenotypes.

[0391] The first two genes of the Type-1 pilus cluster, *fimB* and *fimE* encode recombinases that recognize 9 bp DNA segments of dyad symmetry that flank the invertible *fimA* promoter. Whereas FimB switches pilation “on”, FimE turns the promoter in the “off” orientation. The up- or down-regulation of either *fimB* or *fimE* expression therefore controls the position of the so-called “*fim*-switch” (McClain, M. S., *et al.*, *J. Bacteriol.* 173:5308-5314 (1991); Blomfield, I. C., *et al.*, *J. Bacteriol.* 173:5298-5307 (1991)).

[0392] The two regulatory proteins *fimB* and *fimE* are transcribed from distinct promoters and their transcription was shown to be influenced by a wide range of different factors including the integration host factor (IHF) (Blomfield, I. C., *et al.*, *Mol. Microbiol.* 23:705-717 (1997)) and the leucine-responsive regulatory protein (LRP) (Blomfield, I. C., *et al.*, *J. Bacteriol.* 175:27-36 (1993); Gally, D.

L., *et al.*, *J. Bacteriol.* 175:6186-6193 (1993); Gally, D. L., *et al.*, *Microbiol.* 21:725-738 (1996); Roesch, R. L. & Blomfield, I. C., *Mol. Microbiol.* 27:751-761 (1998)). Mutations in the former lock the bacteria either in "on" or "off" phase, whereas LRP mutants switch with a reduced frequency. In addition, an effect of *leuX* on pilus biogenesis has been shown. This gene is located in the vicinity of the *fim*-genes on the chromosome and codes for the minor leucine tRNA species for the UUG codon. Whereas *fimB* contains five UUG codons, *fimE* contains only two, and enhanced *leuX* transcription might favor FimB over FimE expression (Burghoff, R. L., *et al.*, *Infect. Immun.* 61:1293-1300 (1993); Newman, J. V., *et al.*, *FEMS Microbiol. Lett.* 122:281-287 (1994); Ritter, A., *et al.*, *Mol. Microbiol.* 25:871-882 (1997)).

[0393] Furthermore, temperature, medium composition and other environmental factors were shown to influence the activity of FimB and FimE. Finally, a spontaneous, statistical switching of the *fimA* promoter has been reported. The frequency of this spontaneous switching is approximately  $10^{-3}$  per generation (Eisenstein, B. I., *Science* 214:337-339 (1981); Abraham, S. M., *et al.*, *Proc. Nat. Acad. Sci. USA* 82:5724-5727 (1985)), but is strongly influenced by the above mentioned factors.

[0394] The genes *fimI* and *fimC* are also transcribed from the *fimA* promoter, but directly downstream of *fimA* a DNA segment with a strong tendency to form secondary structure was identified which probably represents a partial transcription terminator (Klemm, P., *Euro. J. Biochem.* 143:395-400 (1984)); and is therefore supposed to severely reduce *fimI* and *fimC* transcription. At the 3' end of *fimC* an additional promoter controls the *fimD* transcription; at the 3' end of *fimD* the last known *fim* promoter is located that regulates the levels of FimF, FimG, and FimH. Thus, all of the minor Type-1 pili proteins are transcribed as a single mRNA (Klemm, P. & Krogfelt, K. A., "Type I fimbriae of *Escherichia coli*," in: *Fimbriae*. Klemm, P. (ed.), CRC Press Inc., (1994) pp. 9-26). This ensures a 1:1:1 stoichiometry on mRNA-level, which is probably maintained on the protein level.

- [0395] In the case of P-pili additional regulatory mechanisms were found when the half-life of mRNA was determined for different P-pilus genes. The mRNA for *papA* was extraordinarily long-lived, whereas the mRNA for *papB*, a regulatory pilus protein, was encoded by short-lived mRNA (Naureckiene, S. & Uhlin, B. E., *Mol. Microbiol.* 21:55-68 (1996); Nilsson, P., *et al.*, *J. Bacteriol.* 178:683-690 (1996)).
- [0396] In the case of Type-1 pili, the gene for the Type-1 pilus chaperone FimC starts with a GTG instead of an ATG codon, leading to a reduced translation efficiency. Finally, analysis of the *fimH* gene revealed a tendency of the *fimH* mRNA to form a stem-loop, which might severely hamper translation. In summary, bacterial pilus biogenesis is regulated by a wide range of different mechanisms acting on all levels of protein biosynthesis.
- [0397] Periplasmic pilus proteins are generally synthesized as precursors, containing a N-terminal signal-sequence that allows translocation across the inner membrane via the Sec-apparatus. After translocation the precursors are normally cleaved by signal-peptidase I. Structural Type-1 pilus subunits normally contain disulfide bonds, their formation is catalyzed by DsbA and possibly DsbC and DsbG gene products.
- [0398] The Type-1 pilus chaperone FimC lacks cysteine residues. In contrast, the chaperone of P-pili, PapD, is the only member of the pilus chaperone family that contains a disulfide bond, and the dependence of P-pili on DsbA has been shown explicitly (Jacob-Dubuisson, F., *et al.*, *Proc. Nat. Acad. Sci. USA* 91:11552-11556 (1994)). PapD does not accumulate in the periplasm of a  $\Delta dsbA$  strain, indicating that the disturbance of the P-pilus assembly machinery is caused by the absence of the chaperone (Jacob-Dubuisson, F., *et al.*, *Proc. Nat. Acad. Sci. USA* 91:11552-11556 (1994)). This is in accordance with the finding that Type-1 pili are still assembled in a  $\Delta dsbA$  strain, albeit to reduced level (Hultgren, S. J., *et al.*, "Bacterial Adhesion and Their Assembly", in: *Escherichia coli and Salmonella*, Neidhardt, F. C. (ed.) ASM Press, (1996) pp. 2730-2756).

- [0399] Type-1 pili as well as P-pili are to 98% made of a single or main structural subunit termed FimA and PapA, respectively. Both proteins have a size of ~15.5 kDa. The additional minor components encoded in the pilus gene clusters are very similar (see Table 1). The similarities in sequence and size of the subunits with the exception of the adhesins suggest that all share an identical folding motif, and differ only with respect to their affinity towards each other. Especially the N- and C-terminal regions of these proteins are well conserved and supposed to play an important role in chaperone/subunit interactions as well as in subunit/subunit interactions within the pilus (Soto, G. E. & Hultgren, S. J., *J. Bacteriol.* 181:1059-1071 (1999)). Interestingly, the conserved N-terminal segment can be found in the middle of the pilus adhesins, indicating a two-domain organization of the adhesins where the proposed C-terminal domain, starting with the conserved motif, corresponds to a structural pilus subunit whereas the N-terminal domain was shown to be responsible for recognition of host cell receptors (Hultgren, S. J., *et al.*, *Proc. Nat. Acad. Sci. USA* 86:4357-4361 (1989); Haslam, D. B., *et al.*, *Mol. Microbiol.* 14:399-409 (1994); Soto, G. E., *et al.*, *EMBO J.* 17:6155-6167 (1998)). The different subunits were also shown to influence the morphological properties of the pili. The removal of several genes was reported to reduce the number of Type-1 or P-pili or to increase their length, (*fimH*, *papG*, *papK*, *fimF*, *fimG*) (Russell, P. W. & Orndorff, P. E., *J. Bacteriol.* 174:5923-5935 (1992); Jacob-Dubuisson, R., *et al.*, *EMBO J.* 12:837-847 (1993); Soto, G. E. & Hultgren, S. J., *J. Bacteriol.* 181:1059-1071 (1999)); combination of the gene deletions amplified these effects or led to a total loss of piliation (Jacob-Dubuisson, R., *et al.*, *EMBO J.* 12:837-847 (1993)).
- [0400] In non-fimbrial adhesive cell organelles also assembled via chaperones/usher systems such as Myf fimbriae and CS3 pili, the conserved C-terminal region is different. This indirectly proves the importance of these C-terminal subunit segments for quaternary interactions (Hultgren, S. J., *et al.*, "Bacterial Adhesion and Their Assembly", in: *Escherichia coli and Salmonella*, Neidhardt, F. C. (ed.) ASM Press, (1996) pp. 2730-2756).



- [0401] Gene deletion studies proved that removal of the pilus chaperones leads to a total loss of piliation in P-pili and Type-1 pili (Lindberg, F., *et al.*, *J. Bacteriol.* 171:6052-6058 (1989); Klemm, P., *Res. Microbiol.* 143:831-838 (1992); Jones, C. H., *et al.*, *Proc. Nat. Acad. Sci. USA* 90:8397-8401 (1993)). Periplasmic extracts of a *ΔfimC* strain showed the accumulation of the main subunit FimA, but no pili could be detected (Klemm, P., *Res. Microbiol.* 143:831-838 (1992)). Attempts to over-express individual P-pilus subunits failed and only proteolytically degraded forms could be detected in the absence of PapD; in addition, the P-pilus adhesin was purified with the inner membrane fraction in the absence of the chaperone (Lindberg, F., *et al.*, *J. Bacteriol.* 171:6052-6058 (1989)). However, co-expression of the structural pilus proteins and their chaperone allowed the detection of chaperone/subunit complexes from the periplasm in the case of the FimC/FimH complex as well as in the case of different Pap-proteins including the adhesin PapG and the main subunit PapA (Tewari, R., *et al.*, *J. Biol. Chem.* 268:3009-3015 (1993); Lindberg, F., *et al.*, *J. Bacteriol.* 171:6052-6058 (1989)). The affinity of chaperone/subunit complexes towards their assembly platform has also been investigated in vitro and was found to differ strongly (Dodson *et al.*, *Proc. Natl. Acad. Sci. USA* 90:3670-3674 (1993)). From these results the following functions were suggested for the pilus chaperones:
- [0402] They are assumed to recognize unfolded pilus subunits, prevent their aggregation and to provide a "folding template" that guides the formation of a native structure.
- [0403] The folded subunits, which after folding display surfaces that allow subunit/subunit interactions, are then expected to be shielded from interacting with other subunits, and to be kept in a monomeric, assembly-competent state.
- [0404] Finally, the pilus chaperones are supposed to allow a triggered release of the subunits at the outer membrane assembly location, and, by doing so with different efficiency, influence the composition and order of the mature pili (see also the separate section below).

- [0405] After subunit release at the outer membrane, the chaperone is free for another round of substrate binding, folding assistance, subunit transport through the periplasm and specific delivery to the assembly site. Since the periplasm lacks energy sources, like ATP, the whole pilus assembly process must be thermodynamically driven (Jacob-Dubuisson, F., *et al.*, *Proc. Nat. Acad. Sci. USA* 91:11552-11556 (1994)). The wide range of different functions attributed to the pilus chaperones would implicate an extremely fine tuned cascade of steps.
- [0406] Several findings, however, are not readily explained with the model of pilus chaperone function outlined above. One example is the existence of multimeric chaperone/subunit complexes (Striker, R. T., *et al.*, *J. Biol. Chem.* 269:12233-12239 (1994)), where one chaperone binds subunit dimers or trimers. It is difficult to imagine a folding template that can be "double-booked". The studies on the molecular details of chaperone/subunit interaction (see below) partially supported the functions summarized above, but also raised new questions.
- [0407] All 31 periplasmic chaperones identified by genetic studies or sequence analysis so far are proteins of approximately 25 kDa with conspicuously high pI values around 10. Ten of these chaperones assist the assembly of rod-like pili, four are involved in the formation of thin pili, ten are important for the biogenesis of atypically thin structures (including capsule-like structures) and two adhesive structures have not been determined so far (Holmgren, A., *et al.*, *EMBO J.* 11:1617-1622 (1992); Bonci, A., *et al.*, *J. Mol. Evolution* 44:299-309 (1997); Smyth, C. J., *et al.*, *FEMS Immun. Med Microbiol.* 16:127-139 (1996); Hung, D. L. & Hultgren, S. J., *J. Struct. Biol.* 124:201-220 (1998)). The pairwise sequence identity between these chaperones and PapD ranges from 25 to 56%, indicating an identical overall fold (Hung, D. L., *et al.*, *EMBO J.* 15:3792-3805 (1996)).
- [0408] The first studies on the mechanism of chaperone/substrate recognition was based on the observation that the C-termini of all known pilus chaperones are extremely similar. Synthetic peptides corresponding to the C-termini of the P-pilus proteins were shown to bind to PapD in ELISA assays (Kuehn, M. J., *et al.*,

*Science* 262:1234-1241 (1993)). Most importantly, the X-ray structures of two complexes were solved in which PapD was co-crystallized with 19-residue peptides corresponding to the C-termini of either the adhesin PapG or the minor pilus component PapK (Kuehn, M. J., *et al.*, *Science* 262:1234-1241 (1993); Soto, G. E., *et al.*, *EMBO J.* 17:6155-6167 (1998)). Both peptides bound in an extended conformation to a  $\beta$ -strand in the N-terminal chaperone domain that is oriented towards the inter-domain cleft, thereby extending a  $\beta$ -sheet by an additional strand. The C-terminal carboxylate groups of the peptides were anchored via hydrogen-bonds to Arg8 and Lys112, these two residues are invariant in the family of pilus chaperones. Mutagenesis studies confirmed their importance since their exchange against alanine resulted in accumulation of non-functional pilus chaperone in the periplasm (Slonim, L. N., *et al.*, *EMBO J.* 11:4747-4756 (1992)). The crystal structure of PapD indicates that neither Arg8 nor Lys112 is involved in stabilization of the chaperone, but completely solvent exposed (Holmgren, A. & Branden, C. I., *Nature* 342:248-251 (1989)). On the substrate side the exchange of C-terminal PapA residues was reported to abolish P-pilus formation, and similar experiments on the conserved C-terminal segment of the P-pilus adhesin PapG prevented its incorporation into the P-pilus (Hultgren, S. J., *et al.*, "Bacterial Adhesion and Their Assembly", in: *Escherichia coli and Salmonella*, Neidhardt, F. C. (ed.) ASM Press, (1996) pp. 2730-2756). All evidence therefore indicated pilus subunit recognition via the C-terminal segments of the subunits.

[0409] A more recent study on C-terminal amino acid exchanges of the P-pilus adhesin PapG gave a more detailed picture. A range of amino acid substitutions at the positions -2, -4, -6, and -8 relative to the C-terminus were tolerated, but changed pilus stability (Soto, G. E., *et al.*, *EMBO J.* 17:6155-6167 (1998)).

[0410] Still, certain problems arise when this model is examined more closely. Adhesive bacterial structures not assembled to rigid, rod-like pili lack the conserved C-terminal segments (Hultgren, S. J., *et al.*, "Bacterial Adhesion and Their Assembly", in: *Escherichia coli and Salmonella*, Neidhardt, F. C. (ed.)

ASM Press, (1996) pp. 2730-2756), even though they are also dependent on the presence of related pilus chaperones. This indicates a different general role for the C-terminal segments of pilus subunits, namely the mediation of quaternary interactions in the mature pilus. Moreover, the attempt to solve the structure of a C-terminal peptide in complex with the chaperone by NMR was severely hampered by the weak binding of the peptide to the chaperone (Walse, B., *et al.*, *FEBS Lett.* 412:115-120 (1997)); whereas an essential contribution of the C-terminal segments for chaperone recognition implies relatively high affinity interactions.

[0411] An additional problem arises if the variability between the different subunits are taken into account. Even though the C-terminal segments are conserved, a wide range of conservative substitutions is found. For example, 15 out of 19 amino acid residues differ between the two peptides co-crystallized with PapD (Soto, G. E., *et al.*, *EMBO J.* 17:6155-6167 (1998)). This has been explained by the kind of interaction between chaperone and substrate, that occurs mainly via backbone interactions and not specifically via side-chain interactions. Then again, the specificity of the chaperone for certain substrates is not readily explained. On the contrary to the former argument, the conserved residues have been taken as a proof for the specificity (Hultgren, S. J., *et al.*, "Bacterial Adhesion and Their Assembly", in: *Escherichia coli and Salmonella*, Neidhardt, F. C. (ed.) ASM Press, (1996) pp. 2730-2756).

[0412] The outer membrane assembly platform, also termed "usher" in the literature, is formed by homo-oligomers of FimD or PapC, in the case of Type-1 and P-pili, respectively (Klemm, P. & Christiansen, G., *Mol. Gen. Genetics* 220:334-338 (1990); Thanassi, D. G., *et al.*, *Proc. Nat. Acad. Sci. USA* 95:3146-3151 (1998)). Studies on the elongation of Type-1 fimbriae by electron microscopy demonstrated an elongation of the pilus from the base (Lowe, M. A., *et al.*, *J. Bacteriol.* 169:157-163 (1987)). In contrast to the secretion of unfolded subunits into the periplasmic space, the fully folded proteins have to be translocated through the outer membrane, possibly in an oligomeric form

(Thanassi, D. G., *et al.*, *Proc. Nat. Acad. Sci. USA* 95:3146-3151 (1998)). This requires first a membrane pore wide enough to allow the passage and second a transport mechanism that is thermodynamically driven (Jacob-Dubuisson, F., *et al.*, *J. Biol. Chem.* 269:12447-12455 (1994)).

[0413] FimD expression alone was shown to have a deleterious effect on bacterial growth, the co-expression of pilus subunits could restore normal growth behavior (Klemm, P. & Christiansen, G., *Mol. Gen. Genetics* 220:334-338 (1990)). Based on this it can be concluded that the ushers probably form pores that are completely filled by the pilus. Electron microscopy on membrane vesicles in which PapC had been incorporated confirmed a pore-forming structure with an inner diameter of 2 nm (Thanassi, D. G., *et al.*, *Proc. Nat. Acad. Sci. USA* 95:3146-3151 (1998)). Since the inner diameter of the pore is too small to allow the passage of a pilus rod, it has been suggested that the helical arrangement of the mature pilus is formed at the outside of the bacterial surface. The finding that glycerol leads to unraveling of pili which then form a protein chain of approximately 2 nm is in good agreement with this hypothesis, since an extended chain of subunits might be formed in the pore as a first step (Abraham, S. N., *et al.*, *J. Bacteriol.* 174:5145-5148 (1992); Thanassi, D. G., *et al.*, *Proc. Nat. Acad. Sci. USA* 95:3146-3151 (1998)). The formation of the helical pilus rod at the outside of the bacterial membrane might then be the driving force responsible for translocation of the growing pilus through the membrane.

[0414] It has also been demonstrated that the usher proteins of Type-1 and P-pili form ternary complexes with chaperone/subunit complexes with different affinities (Dodson, K. W., *et al.*, *Proc. Nat. Acad. Sci. USA* 90:3670-3674 (1993); Saulino, E. T., *et al.*, *EMBO J.* 17:2177-2185 (1998)). This was interpreted as "kinetic partitioning" that allows a defined order of pilus proteins in the pilus. Moreover, it has been suggested that structural proteins might present a binding surface only compatible with one other type of pilus protein; this would be another mechanism to achieve a highly defined order of subunits in the mature pilus (Saulino, E. T., *et al.*, *EMBO J.* 17:2177-2185 (1998)).

B. Production of Type-1 pili from *Escherichia coli*

[0415] *E. coli* strain W3110 was spread on LB (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.5, 1 % agar (w/v)) plates and incubated at 37°C overnight. A single colony was then used to inoculate 5 ml of LB starter culture (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.5). After incubation for 24 hours under conditions that favor bacteria that produce Type-1 pili (37°C, without agitation) 5 shaker flasks containing 1 liter LB were inoculated with one milliliter of the starter culture. The bacterial cultures were then incubated for additional 48 to 72 hours at 37°C without agitation. Bacteria were then harvested by centrifugation (5000 rpm, 4°C, 10 minutes) and the resulting pellet was resuspended in 250 milliliters of 10 mM Tris/HCl, pH 7.5. Pili were detached from the bacteria by 5 minutes agitation in a conventional mixer at 17,000 rpm. After centrifugation for 10 minutes at 10,000 rpm at 4°C the pili containing supernatant was collected and 1 M MgCl<sub>2</sub> was added to a final concentration of 100 mM. The solution was kept at 4°C for 1 hour, and the precipitated pili were then pelleted by centrifugation (10,000 rpm, 20 minutes, 4°C). The pellet was then resuspended in 10 mM HEPES, pH 7.5, and the pilus solution was then clarified by a final centrifugation step to remove residual cell debris.

C. Coupling of FLAG to purified Type-1 pili of *E. coli* using m-Maleimidobenzoyl-N-hydroxysuccinimide ester (sulfo-MBS)

[0416] 600 µl of a 95% pure solution of bacterial Type-1 pili (2 mg/ml) were incubated for 30 minutes at room temperature with the heterobifunctional cross-linker sulfo-MBS (0.5 mM). Thereafter, the mixture was dialyzed overnight against 1 liter of 50 mM Phosphate buffer (pH 7.2) with 150 mM NaCl to remove free sulfo-MBS. Then 500 µl of the derivatized pili (2 mg/ml) were mixed with 0.5 mM FLAG peptide (containing an amino-terminal Cysteine) in the presence of 10 mM EDTA to prevent metal-catalyzed sufhydryloxydation. The non-coupled peptide was removed by size-exclusion-chromatography.

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[0417] Figure 9 depicts an analysis of coupling of the FLAG peptide to type-1 bacterial pili by SDS-PAGE. Lane 1 shows the unreacted pili subunit FimA. Lane 3 shows the purified reaction mixture of the pili with the FLAG peptide. The upper band corresponds to the coupled product, while the lower band corresponds to the unreacted subunit.

#### EXAMPLE 34

##### Construction of an expression plasmid for the expression of Type-1 pili of *Escherichia coli*

[0418] The DNA sequence disclosed in GenBank Accession No. U14003, the entire disclosure of which is incorporated herein by reference, contains all of the *Escherichia coli* genes necessary for the production of type-1 pili from nucleotide number 233947 to nucleotide number 240543 (the *fim* gene cluster). This part of the sequences contains the sequences for the genes *fimA*, *fimI*, *fimC*, *fimD*, *fimF*, *fimG*, and *fimH*. Three different PCRs were employed for the amplification of this part of the *E. coli* genome and subsequent cloning into pUC19 (GenBank Accession Nos. L09137 and X02514) as described below.

[0419] The PCR template was prepared by mixing 10 ml of a glycerol stock of the *E. coli* strain W3110 with 90 ml of water and boiling of the mixture for 10 minutes at 95°C, subsequent centrifugation for 10 minutes at 14,000 rpm in a bench top centrifuge and collection of the supernatant.

[0420] Ten ml of the supernatant were then mixed with 50 pmol of a PCR primer one and 50 pmol of a PCR primer two as defined below. Then 5 ml of a 10X PCR buffer, 0.5 ml of Taq-DNA-Polymerase and water up to a total of 50 ml were added. All PCRs were carried out according to the following scheme: 94°C for 2 minutes, then 30 cycles of 20 seconds at 94°C, 30 seconds at 55°C, and 2 minutes at 72°C. The PCR products were then purified by 1% agarose gel-electrophoresis.

[0421] Oligonucleotides with the following sequences were used to amplify the sequence from nucleotide number 233947 to nucleotide number 235863,

comprising the *fimA*, *fimI*, and *fimC* genes: TAGATGATTACGCCAAGC TTATAATAGAAATAGTTTTTTGAAAGGAAAGCAGCATG (SEQ ID NO:196) and GTCAAAGGCCTTGTCGACGTTATTCCATTACGCCCCGTC ATTTTGG (SEQ ID NO:197).

[0422] These two oligonucleotides also contained flanking sequences that allowed for cloning of the amplification product into puc19 via the restriction sites *HindIII* and *SaI*. The resulting plasmid was termed pFIMAIC (SEQ ID NO:198).

[0423] Oligonucleotides with the following sequences with were used to amplify the sequence from nucleotide number 235654 to nucleotide number 238666, comprising the *fimD* gene: AAGATCTTAAGCTAAGCTTGAATTCTC TGACGCTGATTAACC (SEQ ID NO:199) and ACGTAAAGCATTCTTCT AGACCGCGGATAGTAATCGTGCTATC (SEQ ID NO:200).

[0424] These two oligonucleotides also contained flanking sequences that allowed for cloning of the amplification product into puc19 via the restriction sites *HindIII* and *XbaI*, the resulting plasmid was termed pFIMD (SEQ ID NO:201).

[0425] Oligonucleotides with the following sequences with were used to amplify the sequence from nucleotide number 238575 nucleotide number 240543, comprising the *fimF*, *fimG*, and *fimH* gene: AATTACGTGAGCA AGCTTATGAGAAACAAACCTTTTATC (SEQ ID NO:202) and GACTAAG GCCTTTCTAGATTATTGATAAAACAAAAGTCACGC (SEQ ID NO:203).

[0426] These two oligonucleotides also contained flanking sequences that allowed for cloning of the amplification product into puc19 via the restriction sites *HindIII* and *XbaI*; the resulting plasmid was termed pFIMFGH. (SEQ ID NO:204).

[0427] The following cloning procedures were subsequently carried out to generate a plasmid containing all the above-mentioned *fim*-genes: pFIMAIC was digested *EcoRI* and *HindIII* (2237-3982), pFIMD was digested *EcoRI* and *SstII* (2267-5276), pFIMFGH was digested *SstII* and *HindIII* (2327-2231). The fragments were then ligated and the resulting plasmid, containing all the *fim*-genes necessary for pilus formation, was termed pFIMAICDFGH (SEQ ID NO:205).



## EXAMPLE 35

Construction of an expression plasmid for *Escherichia coli* type-1 pili that lacks the adhesion FimH

- [0428] The plasmid pFIMAICDFGH (SEQ ID NO:205) was digested with KpnI, after which a fragment consisting of nucleotide numbers 8895-8509 was isolated by 0.7% agarose gelelectrophoresis and circularized by self-ligation. The resulting plasmid was termed pFIMAICDFG (SEQ ID NO: 206), lacks the *fimH* gene and can be used for the production of FIMH-free type-1 pili.

## EXAMPLE 36

Expression of type-1 pili using the plasmid pFIMAICDFGH

- [0429] *E. coli* strain W3110 was transformed with pFIMAICDFGH (SEQ ID NO:205) and spread on LB (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.5, 1 % agar (w/v)) plates containing 100 µg/ml ampicillin and incubated at 37°C overnight. A single colony was then used to inoculate 50 ml of LB-glucose starter culture (10 g/L tryptone, 5 g/L yeast extract, 1% (w/v) glucose, 5 g/L NaCl, pH 7.5, 100mg/ml ampicillin). After incubation for 12-16 hours at 37°C at 150 rpm, a 5 liter shaker flasks containing 2 liter LB-glucose was inoculated with 20 milliliter of the starter culture. The bacterial cultures were then incubated for additional 24 hours at 37°C with agitation (150 rpm). Bacteria were then harvested by centrifugation (5000 rpm, 4°C, 10 minutes) and the resulting pellet was resuspended in 250 milliliters of 10 mM Tris/HCl, pH 8. Pili were detached from the bacteria by agitation in a conventional mixer at 17,000 rpm for 5 minutes. After centrifugation for 10 minutes at 10,000 rpm, 1 hour, 4°C the supernatant containing pili was collected and 1 M MgCl<sub>2</sub> was added to a final concentration of 100 mM. The solution was kept at 4°C for 1 hour, and precipitated pili were then pelleted by centrifugation (10,000 rpm, 20 minutes, 4°C). The pellet was then resuspended in 10 mM HEPES, 30 mM EDTA, pH 7.5, for 30 minutes at room temperature, and the pilus solution was then clarified by a final

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centrifugation step to remove residual cell debris. The preparation was then dialyzed against 20 mM HEPES, pH 7.4.

#### EXAMPLE 37

##### Activation of HBcAg-Lys with SPDP

[0430] HBcAg-Lys at a concentration of 15  $\mu$ M was reacted with SPDP at a concentration of 456  $\mu$ M SPDP for 60 minutes at room temperature, resulting in a thirty-fold excess of cross-linker over capsid subunit. The reaction mixture was subsequently loaded on SDS-PAGE for analysis, as shown in Fig. 10. The gel shows that the monomer subunits are cross-linked to dimers and higher-order polymers during the reaction.

#### EXAMPLE 38

##### Multimerization of HBcAg-Lys Upon Reaction With Sulfo-MBS

[0431] HBcAg-Lys at a concentration of 118  $\mu$ M was reacted with 20 mM Sulfo-MBS for 30 minutes at room temperature. As shown in Fig. 11, analysis of the reaction mixture by SDS-PAGE revealed that the HBcAg-Lys monomers internally cross-linked to multimers, as reflected in the absence of a band corresponding to the subunit monomer after cross-linking.

#### EXAMPLE 39

##### Conjugation of HBcAg-Lys-2cys Mut to the FLAG Peptide

[0432] HBcAg-Lys-2cys-Mut at a concentration of 80  $\mu$ M was reacted with sulfa-MBS at a concentration of 8.8 mM for 30 minutes at room temperature, resulting in a 110-fold excess of cross-linker over capsid subunit. The reaction mixture was precipitated two times with 50% ammonium sulfate and resuspended in 20 mM Hepes, 150 mM NaCl, pH 7.4, in a volume equivalent to the reaction volume before precipitation. FLAG peptide containing an N-terminal cysteine was added at a concentration of 1.6 mM and the reaction was allowed to proceed for four

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hours at room temperature. The reaction mixture was subsequently loaded on SDS-PAGE for analysis, and the coupling products are shown in Fig. 12.

#### EXAMPLE 40

##### Conjugation of Pili to the p33 Peptide

[0433] A solution of 1 ml pili at a concentration of 1.5 mg/ml (concentration of the subunit) was reacted with 750  $\mu$ l of a 100 mM Sulfo-MBS solution in 20 mM Hepes, pH 7.4, for 45 minutes at room temperature. The reaction mixture was desalted over a Sephadex G25 column equilibrated with 20 mM Hepes, pH 7.4. Fractions containing pili protein were pooled after analysis by dot blot stained with amidoblack, and 0.6  $\mu$ l of a solution of 100 mM p33 peptide (CGGKAVYNFATM, SEQ ID NO: 175), containing an N-terminal cysteine, in DMSO was added to 100  $\mu$ l of the desalted activated pili and reaction allowed to proceed for four hours at room temperature. The reaction mixture was subsequently analyzed by SDS-PAGE, as shown in Fig. 13.

#### EXAMPLE 41

##### Expression of HBcAg-Lys-2cys-Mut

[0434] The plasmid coding for HBcAg-Lys-2cys-Mut was transformed into *E. coli* K802. A single colony was inoculated into 50 ml LB containing 100 mg/ml ampicillin. The next day, the overnight culture was diluted into 2 L LB medium containing 100 mg/ml ampicillin and grown until  $OD_{600} = 0.6$  at 37°C. Cells were induced with 1 mM IPTG, and grown for another 4 hours at 37°C. The cells were then harvested, and the pellet resuspended in 5 ml of 10 mM  $Na_2HPO_4$ , 0.3 mM NaCl, 10 mM EDTA, 0.25% Tween, pH 7.0. Cells were then disrupted by sonification, and ammonium sulfate was added to a concentration of 20%. The pellet was resuspended in 3 ml PBS buffer, and loaded onto a Sephacryl S-400 column. The protein peak containing the capsid protein corresponding to the size of assembled capsid was collected and loaded onto a hydroxyapatite column for subsequent purification. The protein was eluted in the paththrough fraction.

## EXAMPLE 42

Coupling of DP178c peptide, immunization of mice and determination  
of the IgG subtypes

[0435] DP178c peptide is a fragment of the gp41 protein of HIV virus (Kilby, J.M. *et al.*, *Nature Medicine* 4: 1302-07 (1998)); Wild, C. *et al.*, *Aids Res. Hum. Retroviruses* 9: 1051-53 (1993)).

## A. Coupling of DP178c to Pili

[0436] A solution of 3 ml Pili (2.5 mg/ml) produced as described in Example 33 B was reacted with 500  $\mu$ l of a 100 mM Sulfo-MBS solution for 45 minutes at RT. The reaction mixture was desalted on a Sephadex G25 column equilibrated with 20 mM hepes pH 7.4, and fractions containing pili were pooled. An aliquot of 750  $\mu$ l of the activated pili was diluted in 750  $\mu$ l DMSO, and 2-5  $\mu$ l of a 100 mM DP178c solution in DMSO was added. The reaction was left to react 4 hours at RT, and glucose was added to the reaction mixture to give a final concentration of 0.2%. This solution was then dialyzed against 20 mM Hepes, 0.1% glucose, pH 7.4. The dialyzed coupled pili were centrifuged and loaded on SDS-PAGE for analysis. The result of the coupling reaction is depicted on Figure 14A. The sequence of the DP178c peptide (fragment of the HIV gp41 protein) is CYTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF (SEQ ID No: 176).

## B. Immunization of mice and IgG subtype determination

[0437] 80  $\mu$ g of Pili-DP178c was injected in saline intravenously into female Balb/c mice. These mice were boosted with the same amount of vaccine on day 14 and bled on day 24. DP178-specific IgG in serum was determined on day 24 in a DP178 peptide specific ELISA (DP178c peptide was conjugated to Ribonuclease A using the cross-linker SPDP). In Figure 14B, average results from two mice are shown as optical densities obtained with a 1:50 dilution of the serum.

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## EXAMPLE 43

## Expression and purification of GRA2 polypeptide

[0438] Gra2 is an antigen of *Toxoplasma Gondii*. The 59 c-terminal amino acids of GRA2 with a c-terminal linker of 6 amino acids (GSGGCG, SEQ ID No. 177) were cloned into the pGEX-2T vector (Pharmacia, 27-4801-01). Expression and purification of the GST-fusion protein was carried out as described in the instructions. GST was cleaved from GRA2 with thrombin while the fusion protein was bound to glutathione-sepharose-beads and the reaction stopped after 20 min. with 1 mM PMSF. The sepharose beads were then pelleted by centrifugation and the supernatant containing the GRA2-polypeptide was collected. The solution was then concentrated 10-fold with a Ultrafree-4 centrifugal filter-5K (Millipore, UFV4BCC25). To reduce disulfide bonds which might eventually have formed, the solution was treated with 20 mM DTT 1 h on ice. DTT was removed by loading the protein solution on a PD10 column (Pharmacia). Protein concentration was determined by the Lowry test and concentration of free cysteines in an Ellmann's test. The protein was subsequently analyzed by SDS-PAGE. The GRA2 protein can however not be detected by Commassie staining. A yield of 9 mg GRA2 was obtained from an 8 L culture. The GRA2 amino acid sequence is KEAAGRGMVT VGKLANVES DRSTTTTQAP DSPNGLAETE VPVEPQQRAA HVPVPDFSQSGGCG (SEQ ID No. 178)

## EXAMPLE 44

## Coupling of GRA2 to Pili

## A. Coupling of GRA2 to Pili.

[0439] 6 ml of a 2.5 mg/ml Pili protein solution (produced as described in Example 33 B) were reacted with a 50 fold molar excess of Sulfo-MBS, and desalted over a PD10 column (Pharmacia). 1.5 ml of the reaction mixture were loaded on one column, 1 ml was added and the first 1.5 ml were collected. Fractions containing Pili were identified on a dot blot stained with amidoblack.

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A 300  $\mu\text{g/ml}$  solution of GRA2 was concentrated 100 fold, and 100  $\mu\text{l}$  were reacted with 1.2 ml of the desalted activated Pili solution for 4 hours at RT. The reaction mixture was then dialyzed against 21 of a 20 mM Hepes, 150 mM NaCl, pH 7.2 overnight. Figure 15A shows an analysis of the coupling reaction.

B. Immunization of mice with Pili-GRA2 and IgG subtype determination.

[0440] Mice, were immunized with 50  $\mu\text{g}$  of Pili-GRA2 and boosted on day 14, with the same amount of vaccine. Serum samples were taken on day 0, 6, 14 and 21 after the first immunization. GRA2 specific IgG in serum was determined on day 21 in a GRA2 specific ELISA. Results of two individual mice in each group are shown in Figure 15B. The titer was determined as the dilution of sera resulting in half-maximal optical density ( $\text{OD}_{50}$ ).

#### EXAMPLE 45

##### Coupling of B2- and D2-peptide to Pili

[0441] D2 and B2 peptides are sequences from the OmpC protein of *Salmonella typhi*. It is an outer membrane porin. High level of anti-porin antibodies have been detected in the sera of patients with typhoid fever (Arocklasamy, A. and Krishnaswamy, S., *FEBS Letters* 453: 380-82 (1999)).

A. Coupling of B2- or D2-peptides of the ompC protein of *Salmonella typhi* to Pili

[0442] 6 ml of a 2.5 mg/ml Pili protein solution (produced as described in Example 33 B) were reacted with a 50 fold molar excess of Sulfo-MBS, and desalted over a PD10 column (Pharmacia). 1.5 ml of the reaction mixture were loaded on one column, 1 ml was added, and the first 1.5 ml were collected. Fractions containing Pili were identified on a dot blot stained with amidoblack. An aliquot of 5  $\mu\text{l}$  of a 100 mM solution of peptide was reacted with 2.6 ml of the desalted activated Pili solution for 4 hours at RT. The reaction mixture was then

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dialyzed against 21 of a 20 mM Hepes, 150 mM NaCl, pH 7.2 overnight. Figure 16A shows an analysis of the coupling reaction. The sequence of the D2 peptide is CGG TSN GSN PST SYG FAN (SEQ ID No. 179). The sequence of the B2 peptide is CGG DIS NGY GAS YGD NDI (SEQ ID No. 180).

B. Immunization of mice with Pili-B2 and IgG subtype determination.

[0443] Mice were immunized interaperitoneally in female Balb/c mice with 50  $\mu$ g of Pili-B2 in saline and boosted on day 14 with the same amount of vaccine, and bled on day 33. B2-peptide specific IgG in serum was determined on day 33 in a B2-specific ELISA (B2 peptide was conjugated to Ribonuclease A with the cross-linker SPDP). Average of the results of two individual mice are shown in Figure 16B.

#### EXAMPLE 46

[0444] The muTNFa peptide, comprising amino acids 22-33 of TNFa protein was coupled to Pili as described in Example 42, except that no glucose was added during the final dialysis step, where the reaction solution was dialyzed against 20 mM Hepes, pH 7.4 only. Two Balb/c female mice, 8 days of age were immunized intravenously with 100  $\mu$ g of Pili-muTNFa each. These mice were boosted at day 14 with the same amount of vaccine, and bled on day 20. IgG specific for native TNFa protein in serum was detected at day 20 in an ELISA. As a control, preimmune sera of two mice were assayed for binding to TNFa protein. See Figure 17. The sequence of the muTNFa peptide was CGGVEEQLEWLSQR (SEQ ID No. 181).

#### EXAMPLE 47

A. Preparation of bacterial type-1 pili coupled to TNF peptides

[0445] Two peptides comprising murine TNFa sequences were designed. Peptide 3' murine TNFa II (3'-TNFa II) was SSQNSSDKPVAHVVANHGVGGC

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(SEQ ID No. 182). Peptide 5' murine TNF $\alpha$  II (5' TNF $\alpha$  II) was CSSQNSSDKPVAHVVANHGV (SEQ ID No. 183). The peptides 5'-TNF $\alpha$  II and 3'-TNF $\alpha$  II were coupled to bacterial type-1 pili as follows. An aliquot of 1 ml of a Pili solution (2.5 mg/ml) was reacted with 503  $\mu$ l of a 100 mM Sulfo-NMS solution for 45 minutes at RT. The reaction mixture was desalted over a desalting column previously saturated with Pili protein and equilibrated in 20 mM Hepes, pH 7.4. The fractions containing protein were pooled. An aliquot of 1 ml of desalted Pili was mixed with 1.56  $\mu$ l of peptide (100 mM in DMSO), and the reaction left to proceed for 4 hours at RT. The reaction solution was then dialyzed overnight against 20 mM Hepes, 150 mM NaCl, pH 7.4 in the cold. See Figure 18A.

B. Immunization and detection of antibodies specific for native TNF $\alpha$  and the 3' TNFII and 5' TNFII peptides

[0446] Balb/c mice were vaccinated intraperitoneally with 30  $\mu$ g protein in saline, on day 0, 14 and 33. IgG antibodies specific for native TNF $\alpha$  protein (Fig. 18B) and for the 3' TNFII and 5' TNFII peptides (Fig. 18C) were measured in a specific ELISA.

1. Native TNF $\alpha$  ELISA

[0447] 2  $\mu$ g/ml native TNF $\alpha$  protein was coated on ELISA plates. Sera were added at different dilutions and bound IgG was detected with a horseradish peroxidase-conjugated anti-murine IgG antibody. Results from four individual mice are shown on day 21 and day 43.

2. Anti peptide ELISA

[0448] IgG antibodies specific for the 3' TNFII and 5' TNFII peptides were measured in a specific ELISA 10  $\mu$ g/ml Ribonuclease A coupled to 3' TNFII or 5' TNFII peptide was coated on ELISA plates. Sera were added at different



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dilutions and bound IgG was detected with a horseradish peroxidase-conjugated anti-murine IgG antibody. Results from four individual mice are shown on day 21.

C. Analysis of sera from mice immunized under B.: IgG subtype determination

[0449] Sera from the immunized mice described under B. were taken on day 50. Antibodies specific for the TNF peptides described under A. were measured in a specific ELISA on day 50. RNase coupled to the corresponding TNF peptide was coated on ELISA plates at a concentration of 10  $\mu\text{g/ml}$ . Sera were added at different dilutions and bound antibody was detected with horse radish peroxidase-conjugated anti-murine antibodies. See Figure 18D.

EXAMPLE 48

Coupling of Pili to M2 peptide, immunization of mice, and IgG subtype determination

[0450] M2 peptide was coupled to pili as described in Example 47. The peptide was reacted at a fivefold molar excess with the activated Pili. Female Balb/c mice were injected with 50  $\mu\text{g}$  Pili-M2 in saline subcutaneously. Mice were boosted with the same amount of vaccine on day 14 and bled on day 27, M2 specific IgG in serum was determined on day 27 in a M2-specific ELISA (peptide conjugated to Ribonuclease A with the cross-linker SPDP for coating). See Figures 19A and 19B.

EXAMPLE 49

Immunization of mice with HbcAg-Lys-2cys-Mut coupled to the Flag peptide, and IgG subtype determination

[0451] Flag peptide (SEQ ID NO: 147) was coupled to HBcAg-Lys-2cys-Mut as described in Example 39. Two Balb/c mice were vaccinated intravenously with

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50  $\mu$ g HBc-Ag-Lys-2cys-Mut -Flag. On day 14 mice were boosted with the same amount of vaccine and bled on day 40, Flag-specific antibodies (Flag peptide was conjugated to Ribonuclease A with the cross-linker SPDP for coating) in serum were measured on day 40 in a specific ELISA. ELISA plates were coated with 10  $\mu$ g/ml RNase coupled to Flag peptide and serum was added at a 1:40 dilution. Bound antibodies were detected with peroxidase conjugate isotype-specific IgG. Results from the two mice are shown as ELISA titers in Figure 20.

#### EXAMPLE 50

##### Purification of Type-1 Pili of *Eschericia coli*

- [0452] Isolated Type-1 pili of *Eschericia coli* prepared as described in Example 33B were precipitated with ammonium sulfate, added to a final concentration of 0.5 M, at 4°C for 30 minutes. The pili were then pelleted by centrifugation at 20,000 rpm for 15 min at 4°C and the pellet was resuspended in 25 ml of 20 mM HEPES buffer, pH 7.3. The precipitation step was repeated once, and the final sample was resuspended in 9 ml of 20 mM HEPES, pH 7.3 and finally dialyzed against the same buffer to remove residual ammonium sulfate. The pili were subsequently purified on an SR-400 size exclusion chromatography column (20 mM HEPES, pH 7.3) and the pili containing fractions were collected and pooled.
- [0453] All patents and publications referred to herein are expressly incorporated by reference.
- [0454] The entire disclosure of U.S. Application No. 09/449,631, filed November 30, 1999, is herein incorporated by reference. All publications and patents mentioned hereinabove are hereby incorporated in their entireties by reference.

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WHAT IS CLAIMED IS:

1. A composition comprising a bacterial pilus to which an antigen or antigenic determinant has been attached by a covalent bond.
2. The composition of claim 1, wherein said covalent bond is not a peptide bond.
3. The composition of claim 1, wherein said bacterial pilus is a Type-1 pilus of *Escherichia coli*.
4. The composition of claim 1, wherein pilin subunits of said Type-1 pilus comprises the amino acid sequence shown in SEQ ID NO:146 or a sequence having at least 65, 70, 75, 80, 85, 90 or 95% sequence identity to SEQ ID NO:146.
5. The composition of claim 1, wherein said bacterial pilus and said antigen or antigen determinant are attached via a non-naturally occurring attachment.
6. The composition of claim 1, wherein said attachment comprises an organizer comprising at least one first attachment site, and wherein said organizer is connected to said pilus by at least one covalent bond.
7. The composition of claim 6, wherein said organizer is a polypeptide or a residue thereof, and wherein said second attachment site is a polypeptide or a residue thereof.
8. The composition of claim 6, wherein said first and/or a second attachment sites comprise:

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- (a) an antigen and an antibody or antibody fragment thereto;
- (b) biotin and avidin;
- (c) strepavidin and biotin;
- (d) a receptor and its ligand;
- (e) a ligand-binding protein and its ligand;
- (f) interacting leucine zipper polypeptides;
- (g) an amino group and a chemical group reactive thereto;
- (h) a carboxyl group and a chemical group reactive thereto;
- (i) a sulfhydryl group and a chemical group reactive thereto;

or

- (j) a combination thereof.

9. The composition of claim 1, wherein said bacterial pilus and said antigen or antigenic determinant are attached by an attachment comprising interacting leucine zipper polypeptides.

10. The composition of claim 5, wherein interacting leucine zipper polypeptides are JUN and/or FOS leucine zipper polypeptides.

11. A composition comprising a bacterial pilin polypeptide to which an antigen or antigenic determinant has been attached by a covalent bond.

12. The composition of claim 11, wherein said covalent bond is not a peptide bond.

13. The composition of claim 11, wherein said polypeptide is from a Type-1 pilus of *Escherichia coli*.

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14. The composition of claim 11, wherein said bacterial pilin polypeptide comprises the amino acid sequence shown in SEQ ID NO:146 or a sequence having at least 65, 70, 75, 80, 85, 90 or 95% sequence identity to SEQ ID NO:146.

15. The composition of claim 11, wherein said bacterial pilin polypeptide and said antigen or antigenic determinant are attached by a non-naturally occurring attachment.

16. The composition of claim 11, wherein said attachment comprises an organizer comprising at least one first attachment site, and wherein said organizer is connected to said pilus by at least one covalent bond.

17. The composition of claim 16, wherein said organizer is a polypeptide or a residue thereof, and wherein said second attachment site is a polypeptide or a residue thereof.

18. The composition of claim 11, wherein said first and/or a second attachment sites comprise:

- (a) an antigen and an antibody or antibody fragment thereto;
- (b) biotin and avidin;
- (c) strepavidin and biotin;
- (d) a receptor and its ligand;
- (e) a ligand-binding protein and its ligand;
- (f) interacting leucine zipper polypeptides;
- (g) an amino group and a chemical group reactive thereto;
- (h) a carboxyl group and a chemical group reactive thereto;
- (i) a sulfhydryl group and a chemical group reactive thereto;

or

- (j) a combination thereof.

19. The composition of claim 15, wherein said attachment comprises interacting leucine zipper polypeptides.

20. The composition of claim 13, wherein said interacting leucine zipper polypeptides are JUN and/or FOS leucine zipper polypeptides.

21. A composition comprising:

- (a) a non-natural molecular scaffold comprising:
  - (i) a core particle selected from the group consisting of:
    - (1) a bacterial pilus or pilin protein; and
    - (2) a recombinant form of a bacterial pilus or pilin protein; and
  - (ii) an organizer comprising at least one first attachment site,  
wherein said organizer is connected to said core particle by at least one covalent bond; and
- (b) an antigen or antigenic determinant with at least one second attachment site, said second attachment site being selected from the group consisting of:
  - (i) an attachment site not naturally occurring with said antigen or antigenic determinant; and
  - (ii) an attachment site naturally occurring with said antigen or antigenic determinant,  
wherein said second attachment site is capable of association through at least one non-peptide bond to said first attachment site; and  
wherein said antigen or antigenic determinant and said scaffold interact through said association to form an ordered and repetitive antigen array.

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22. The composition of claim 21, wherein said organizer is a polypeptide or residue thereof, and wherein said second attachment site is a polypeptide or residue thereof.

23. The composition of claim 21, wherein said first and/or said second attachment sites comprise:

- (a) an antigen and an antibody or antibody fragment thereto;
- (b) biotin and avidin;
- (c) strepavidin and biotin;
- (d) a receptor and its ligand;
- (e) a ligand-binding protein and its ligand;
- (f) interacting leucine zipper polypeptides;
- (g) an amino group and a chemical group reactive thereto;
- (h) a carboxyl group and a chemical group reactive thereto;
- (i) a sulfhydryl group and a chemical group reactive thereto;

or

- (j) a combination thereof.

24. The composition of claim 21, wherein said first and/or said second attachment sites comprise interacting leucine zipper polypeptides.

25. The composition of claim 21, wherein said bacterial pilus is a Type-1 pilus of *Escherichia coli*.

26. The composition of claim 21, wherein pilus subunits of said type-1 pilus comprise the amino acid sequence of SEQ ID No. 146 or a sequence having at least 65, 70, 75, 80, 85, 90 or 95% sequence identity to SEQ ID NO:146.

27. The composition of claim 26, wherein said interacting leucine zipper polypeptides are the JUN and/or FOS leucine zipper polypeptides.

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28. A composition comprising:
- (a) a non-natural molecular scaffold comprising:
    - (i) a virus-like particle that is a dimer or a multimer of a polypeptide comprising amino acids 1-147 of SEQ ID NO:158 as core particle or a sequence having at least 65, 70, 75, 80, 85, 90 or 95% sequence identity to SEQ ID NO:158; and
    - (ii) an organizer comprising at least one first attachment site,
- wherein said organizer is connected to said core particle by at least one covalent bond; and
- (b) an antigen or antigenic determinant with at least one second attachment site, said second attachment site being selected from the group consisting of:
    - (i) an attachment site not naturally occurring with said antigen or antigenic determinant; and
    - (ii) an attachment site naturally occurring with said antigen or antigenic determinant,
- wherein said second attachment site is capable of association through at least one non-peptide bond to said first attachment site; and
- wherein said antigen or antigenic determinant and said scaffold interact through said association to form an ordered and repetitive antigen array.

29. The composition of claim 28, wherein said organizer is a polypeptide or residue thereof; and wherein said second attachment site is a polypeptide or residue thereof.

30. The composition of claim 28, wherein said first and/or said second attachment sites comprise:

- (a) an antigen and an antibody or antibody fragment thereto;
- (b) biotin and avidin;



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- (c) strepavidin and biotin;
- (d) a receptor and its ligand;
- (e) a ligand-binding protein and its ligand;
- (f) interacting leucine zipper polypeptides;
- (g) an amino group and a chemical group reactive thereto;
- (h) a carboxyl group and a chemical group reactive thereto;
- (i) a sulfhydryl group and a chemical group reactive thereto;

or

- (j) a combination thereof.

31. The composition of claim 30, wherein said first attachment site is an amino group and said second attachment site is a sulfhydryl group.

32. The composition of claim 30, wherein said virus-like particle and said antigen or antigenic determinant are attached by an attachment comprising interacting leucine zipper polypeptides.

33. The composition of claim 32, wherein said interacting leucine zipper polypeptides are JUN and/or FOS polypeptides.

34. A composition comprising:

- (a) a non-natural molecular scaffold comprising:

- (i) Hepatitis B virus capsid protein comprising an amino acid sequence selected from the group consisting of:

- (1) the amino acid sequence of SEQ ID NO:89;
    - (2) the amino acid sequence of SEQ ID NO:90;
    - (3) the amino acid sequence of SEQ ID NO:93;
    - (4) the amino acid sequence of SEQ ID NO:98;
    - (5) the amino acid sequence of SEQ ID NO:99;

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- (6) the amino acid sequence of SEQ ID NO:  
102;
- (7) the amino acid sequence of SEQ ID NO:  
104;
- (8) the amino acid sequence of SEQ ID  
NO:105;
- (9) the amino acid sequence of SEQ ID  
NO:106;
- (10) the amino acid sequence of SEQ ID  
NO:119;
- (11) the amino acid sequence of SEQ ID  
NO:120;
- (12) the amino acid sequence of SEQ ID  
NO:123;
- (13) the amino acid sequence of SEQ ID  
NO:125;
- (14) the amino acid sequence of SEQ ID  
NO:131;
- (15) the amino acid sequence of SEQ ID  
NO:132;
- (16) the amino acid sequence of SEQ ID  
NO:134;
- (17) the amino acid sequence of SEQ ID  
NO:157; and
- (18) the amino acid sequence of SEQ ID  
NO:158; and

(ii) an organizer comprising at least one first attachment  
site,

wherein said organizer is connected to said core particle by at least one  
covalent bond; and

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(b) an antigen or antigenic determinant with at least one second attachment site, said second attachment site being selected from the group consisting of:

(i) an attachment site not naturally occurring with said antigen or antigenic determinant; and

(ii) an attachment site naturally occurring with said antigen or antigenic determinant,

wherein said second attachment site is capable of association through at least one non-peptide bond to said first attachment site; and

wherein said antigen or antigenic determinant and said scaffold interact through said association to form an ordered and repetitive antigen array.

35. The composition of claim 34, wherein said organizer is a polypeptide or residue thereof,

wherein said second attachment site is a polypeptide or residue thereof, and

wherein said first attachment site is a lysine residue and said second attachment site is a cysteine residue.

36. The composition of claim 34, wherein one or more cysteine residues of said Hepatitis B virus capsid protein have been either deleted or substituted with another amino acid residue.

37. The composition of claim 34, wherein said first and/or said second attachment sites comprise:

- (a) an antigen and an antibody or antibody fragment thereto;
- (b) biotin and avidin;
- (c) strepavidin and biotin;
- (d) a receptor and its ligand;
- (e) a ligand-binding protein and its ligand;

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- (f) interacting leucine zipper polypeptides;
- (g) an amino group and a chemical group reactive thereto;
- (h) a carboxyl group and a chemical group reactive thereto;
- (i) a sulfhydryl group and a chemical group reactive thereto;

or

- (j) a combination thereof.

38. The composition of claim 36, wherein the cysteine residues corresponding to amino acids 48 and 107 in SEQ ID NO:134 have been either deleted or substituted with another amino acid residue.

39. The composition of claim 37, wherein said Hepatitis B virus capsid protein and said antigen or antigenic determinant are attached by an attachment comprising interacting leucine zipper polypeptides.

40. The composition of claim 39, wherein said interacting leucine zipper polypeptides are FOS and/or JUN polypeptides.

41. The composition of any one of claims 28, 34, 35, 36 and 38, wherein said antigen is selected from the group consisting of:

- (a) an antigen suited to induce an immune response against bacteria,
- (b) an antigen suited to induce an immune response against viruses,
- (c) an antigen suited to induce an immune response against parasites,
- (d) an antigen suited to induce an immune response against cancer cells,
- (e) an antigen suited to induce an immune response against allergens,

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- (f) an antigen suited to induce an immune response in a farm animals, and
- (g) a protein suited to induce an immune response in a pet.

42. The composition of claim 41, wherein the antigen is a protein, polypeptide, or a fragment thereof.

43. The composition of claim 47, wherein said antigen induces an immune response against one or more allergens.

44. The composition of claim 47, wherein said antigen is:

- (a) a recombinant protein of HIV,
- (b) a recombinant protein of Influenza virus,
- (c) a recombinant protein of Hepatitis C virus,
- (d) a recombinant protein of Toxoplasma,
- (e) a recombinant protein of Plasmodium falciparum,
- (f) a recombinant protein of Plasmodium vivax,
- (g) a recombinant protein of Plasmodium ovale,
- (h) a recombinant protein of Plasmodium malariae,
- (i) a recombinant protein of breast cancer cells,
- (j) a recombinant protein of kidney cancer cells,
- (k) a recombinant protein of prostate cancer cells,
- (l) a recombinant protein of skin cancer cells,
- (m) a recombinant protein of brain cancer cells,
- (n) a recombinant protein of leukemia cells,
- (o) a recombinant profiling,
- (p) a recombinant protein of bee sting allergy,
- (q) a recombinant protein of nut allergy,
- (r) a recombinant protein of food allergies,
- (s) a recombinant protein of asthma, or

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- (t) a recombinant protein of Chlamydia.

45. The composition of any one of claims 1, 11 and 21, wherein said antigen is selected from the group consisting of:

- (a) an antigen suited to induce an immune response against bacteria,
- (b) an antigen suited to induce an immune response against viruses,
- (c) an antigen suited to induce an immune response against parasites,
- (d) an antigen suited to induce an immune response against cancer cells,
- (e) an antigen suited to induce an immune response in a farm animals, and
- (f) an antigen suited to induce an immune response in a pet, and
- (g) any other antigen involved in a pathophysiological context.

46. The composition of claim 45, wherein the antigen is a protein, a polypeptide, or a fragment thereof.

47. The composition of any one of claims 1, 11 or 21, wherein said antigen is:

- (a) a recombinant protein of HIV,
- (b) a recombinant protein of Influenza virus,
- (c) a recombinant protein of Hepatitis C virus,
- (d) a recombinant protein of Toxoplasma,
- (e) a recombinant protein of Plasmodium falciparum,
- (f) a recombinant protein of Plasmodium vivax,
- (g) a recombinant protein of Plasmodium ovale,

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- (h) a recombinant protein of *Plasmodium malariae*,
- (i) a recombinant protein of breast cancer cells,
- (j) a recombinant protein of kidney cancer cells,
- (k) a recombinant protein of prostate cancer cells,
- (l) a recombinant protein of skin cancer cells,
- (m) a recombinant protein of brain cancer cells,
- (n) a recombinant protein of leukemia cells,
- (o) a recombinant profiling,
- (p) a recombinant protein of *Chlamydia*.

48. A pharmaceutical composition comprising the composition of any one of claims 1, 11, 21, 28, 34, 35, 36, 38, 41 or 44, and a pharmaceutically acceptable carrier.

49. A vaccine composition comprising the composition of any one of claims 1, 11, 21, 28, 34, 35, 36, 38, 41 or 44.

50. The vaccine composition of claim 49, further comprising at least one adjuvant.

51. A method of immunizing, comprising administering to a subject the vaccine composition of claim 49 or 50.

52. The method of claim 51, wherein said administering produces an immune response.

53. The method of claim 51, wherein said administering produces a humoral immune response.

54. The method of claim 51, wherein said administering produces a cellular immune response.

55. The method of claim 51, wherein said administering produces a humoral immune response and a cellular immune response.

56. The method of claim 51, wherein said administering produces a protective immune response.

57. A method of making the composition of claim 1, comprising combining said pilus and said antigen or antigenic determinant, wherein said pilus and said antigen or antigenic determinant interact to form an antigen array.

58. The method of claim 57, wherein said antigen array is ordered and/or repetitive.

59. A method of making the composition of claim 11, comprising combining said pilin polypeptide and said antigen or antigenic determinant, wherein said pilin polypeptide and said antigen or antigenic determinant interact to form an antigen array.

60. The method of claim 61, wherein said antigen array is ordered and/or repetitive.

61. A method of making the composition of claim 21, 28, 34, 35, 36 or 38, comprising combining said non-natural molecular scaffold and said antigen or antigenic determinant, wherein said non-natural molecular scaffold and said antigen or antigenic determinant interact to form an antigen array.



62. The method of claim 61, wherein said antigen array is ordered and/or repetitive.

63. A composition comprising:

(a) a non-natural molecular scaffold comprising:

(i) a core particle selected from the group consisting

of:

(1) a bacterial pilus; and

(2) a recombinant form of a bacterial pilus or

pilin protein; and

(ii) an organizer comprising at least one first attachment

site,

wherein said organizer is connected to said core particle by at least one covalent bond; and

(b) an antigen or antigenic determinant with at least one second attachment site, said second attachment site being selected from the group consisting of:

(i) an attachment site not naturally occurring with said antigen or antigenic determinant; and

(ii) an attachment site naturally occurring with said antigen or antigenic determinant,

wherein said second attachment site is capable of association through at least one non-peptide bond to said first attachment site;

wherein said antigen or antigenic determinant and said scaffold interact through said association to form an ordered and repetitive antigen array, and

wherein said antigen or antigenic determinant is selected from the group consisting of an influenza M2 peptide, the GRA2 polypeptide, the DP178c peptide, the tumor necrosis factor polypeptide, a tumor necrosis factor peptide, the B2 peptide, the D2 peptide, and the A $\beta$  peptide.

64. The composition of claim 63, wherein said antigen or antigenic determinant is the influenza M2 peptide or variants thereof.

65. The composition of claim 63, wherein said antigen or antigenic determinant is the GRA2 polypeptide.

66. The composition of claim 63, wherein said antigen or antigenic determinant is the DP178c peptide.

67. The composition of claim 63, wherein said antigen or antigenic determinant is the tumor necrosis factor polypeptide.

68. The composition of claim 63, wherein said antigen or antigenic determinant is a tumor necrosis factor peptide.

69. The composition of claim 63, wherein said antigen or antigenic determinant is the B2 peptide.

70. The composition of claim 63, wherein said antigen or antigenic determinant is the D2 peptide.

71. The composition of claim 63, wherein said antigen or antigenic determinant is the A $\beta$  peptide.

72. The composition of claim 63, wherein said organizer is a polypeptide or residue thereof; and wherein said second attachment site is a polypeptide or residue thereof.

73. The composition of claim 63, wherein said first and/or said second attachment sites comprise:

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- (a) an antigen and an antibody or antibody fragment thereto;
- (b) biotin and avidin;
- (c) strepavidin and biotin;
- (d) a receptor and its ligand;
- (e) a ligand-binding protein and its ligand;
- (f) interacting leucine zipper polypeptides;
- (g) an amino group and a chemical group reactive thereto;
- (h) a carboxyl group and a chemical group reactive thereto;
- (i) a sulfhydryl group and a chemical group reactive thereto;

or

- (j) a combination thereof.

74. The composition of claim 63, wherein said first and/or said second attachment sites comprise interacting leucine zipper polypeptides.

75. The composition of claim 63, wherein said bacterial pilus is a Type-1 pilus of *Escherichia coli*.

76. The composition of claim 63, wherein pilus subunits of said type-1 pilus comprise the amino acid sequence of SEQ ID No. 146 or a sequence having at least 65, 70, 75, 80, 85, 90 or 95% sequence identity to SEQ ID NO:146.

77. The composition of claim 63, wherein said interacting leucine zipper polypeptides are the JUN and/or FOS leucine zipper polypeptides.

78. A vaccine composition comprising the composition of claim 63 or claim 43.

79. A method of immunizing, comprising administering to a subject the vaccine composition of claim 49 or 50.

80. The method of claim 79, wherein said administering produces an immune response.

81. A method of making the composition of claim 63, comprising combining said non-natural molecular scaffold and said antigen or antigenic determinant, wherein said non-natural molecular scaffold and said antigen or antigenic determinant interact to form an antigen array.

82. The method of claim 81, wherein said antigen array is ordered and/or repetitive.

83. A method of immunizing, comprising administering the composition of any one of claims 1, 11, 21, 49 or 50 to a subject, wherein for inducing a Th2 response, wherein said administering produces a Th2 response that is specific for said antigen or antigenic determinant.

84. The method of claim 83, wherein antibodies specific for said antigen or antigenic determinant of a subtype corresponding to the Th2 subtype are induced in the subject.

85. The method of claim 83, wherein the subject does not generate a Th1 response that is specific for said pilin, said pilin polypeptide, or said antigen or antigenic determinant.

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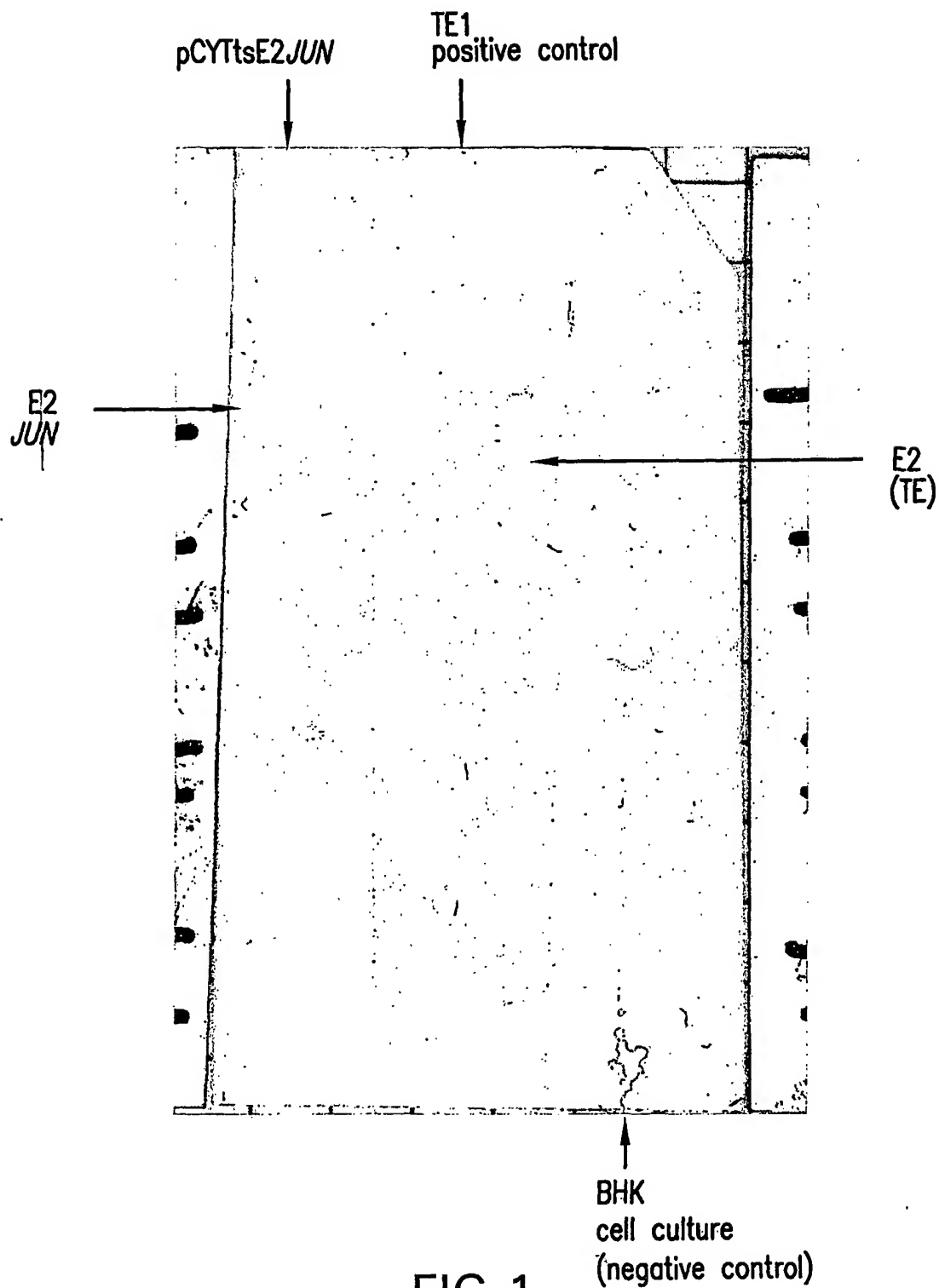


FIG.1

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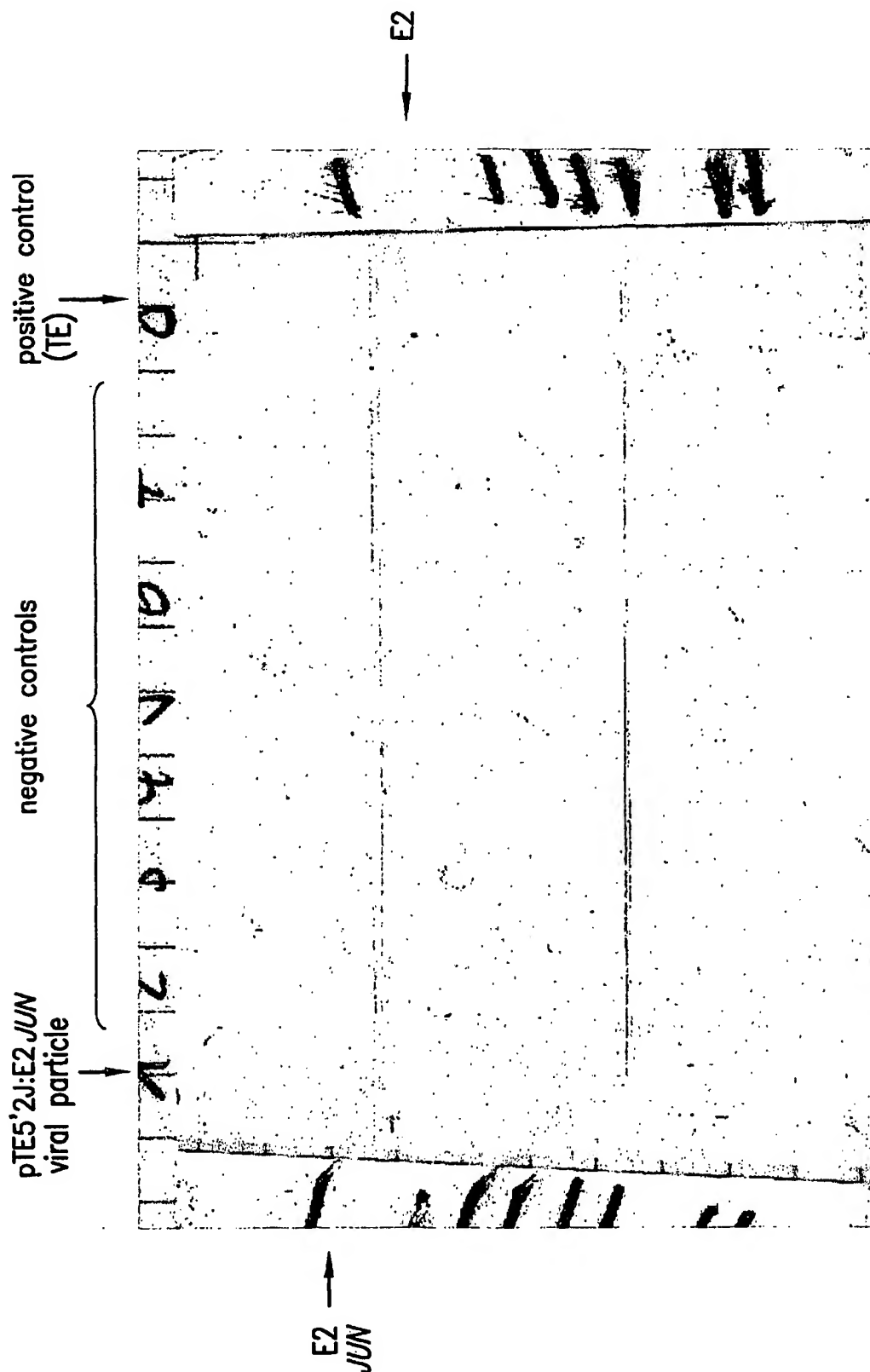


FIG.2

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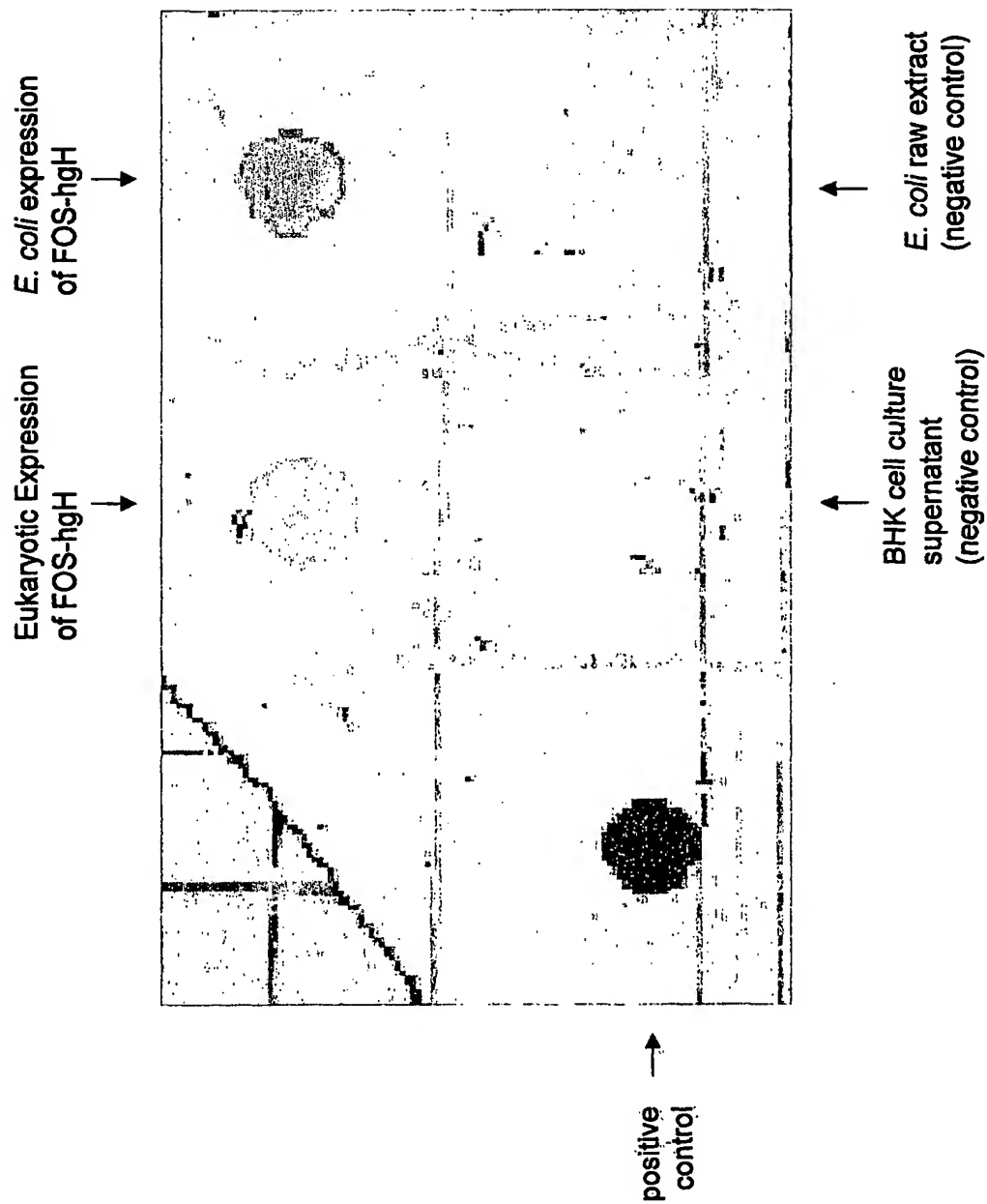


FIG.3

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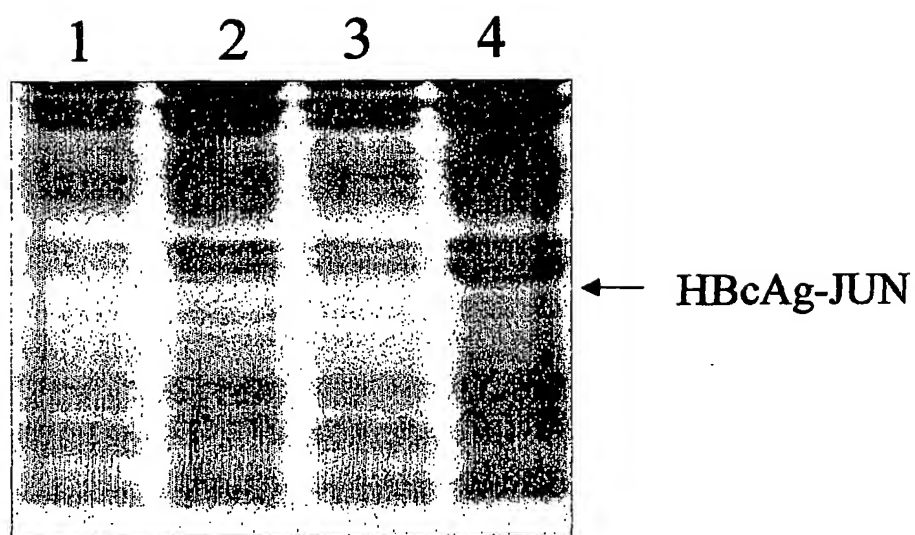


FIG.4



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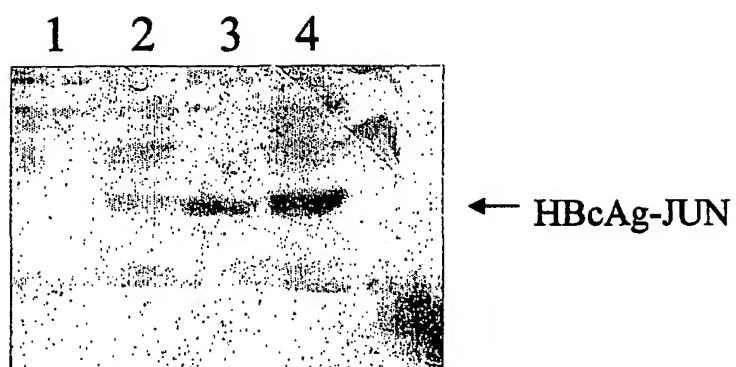


FIG.5

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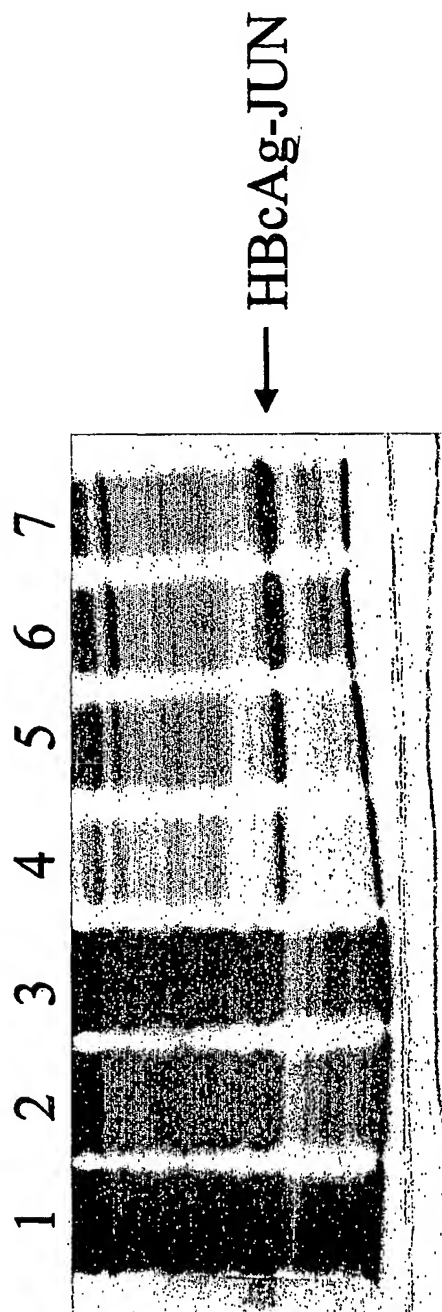


FIG.6

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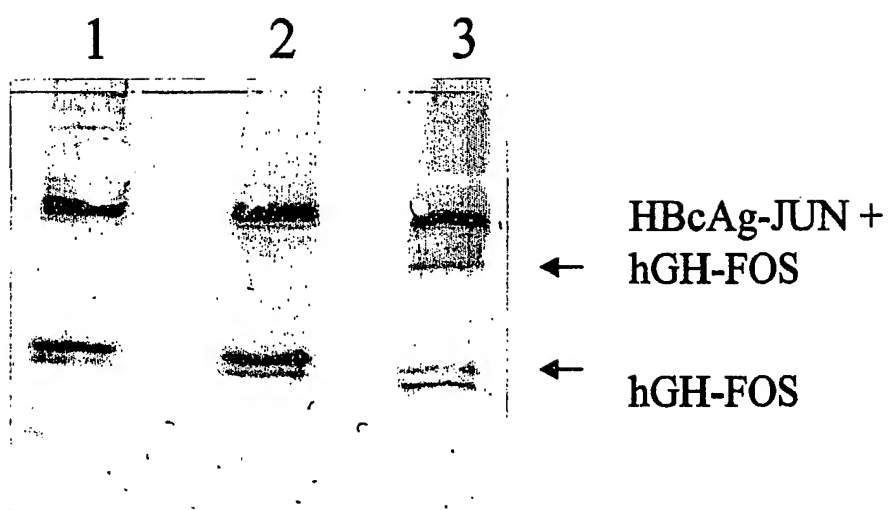


FIG.7

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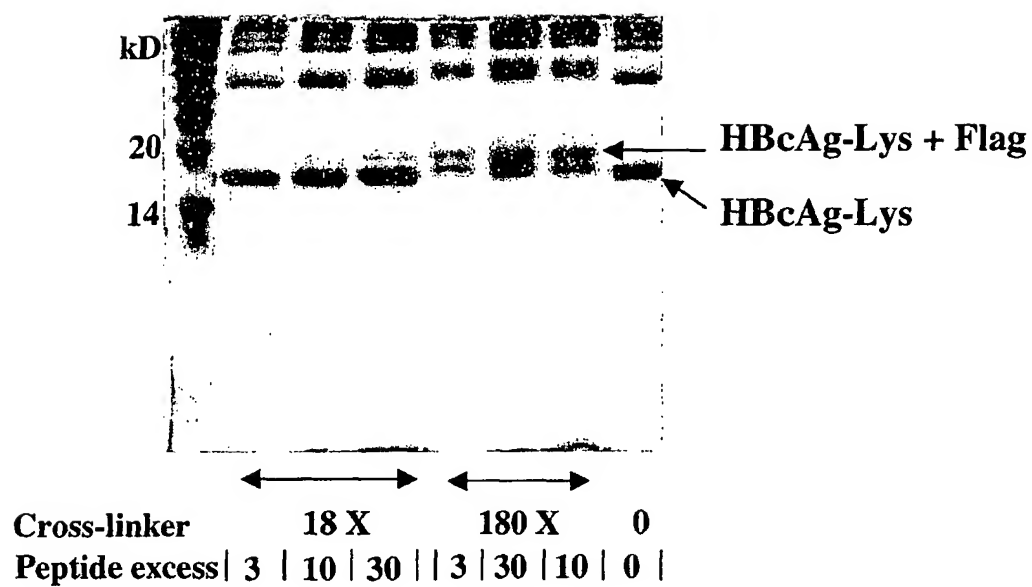


FIG.8

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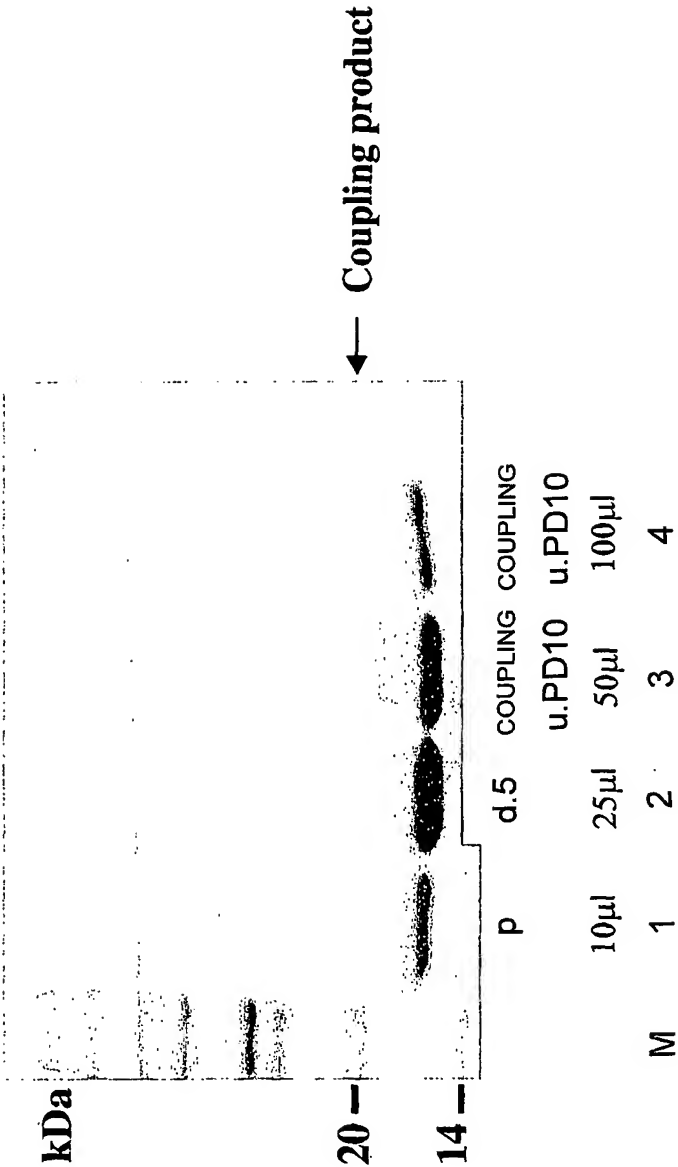


FIG.9

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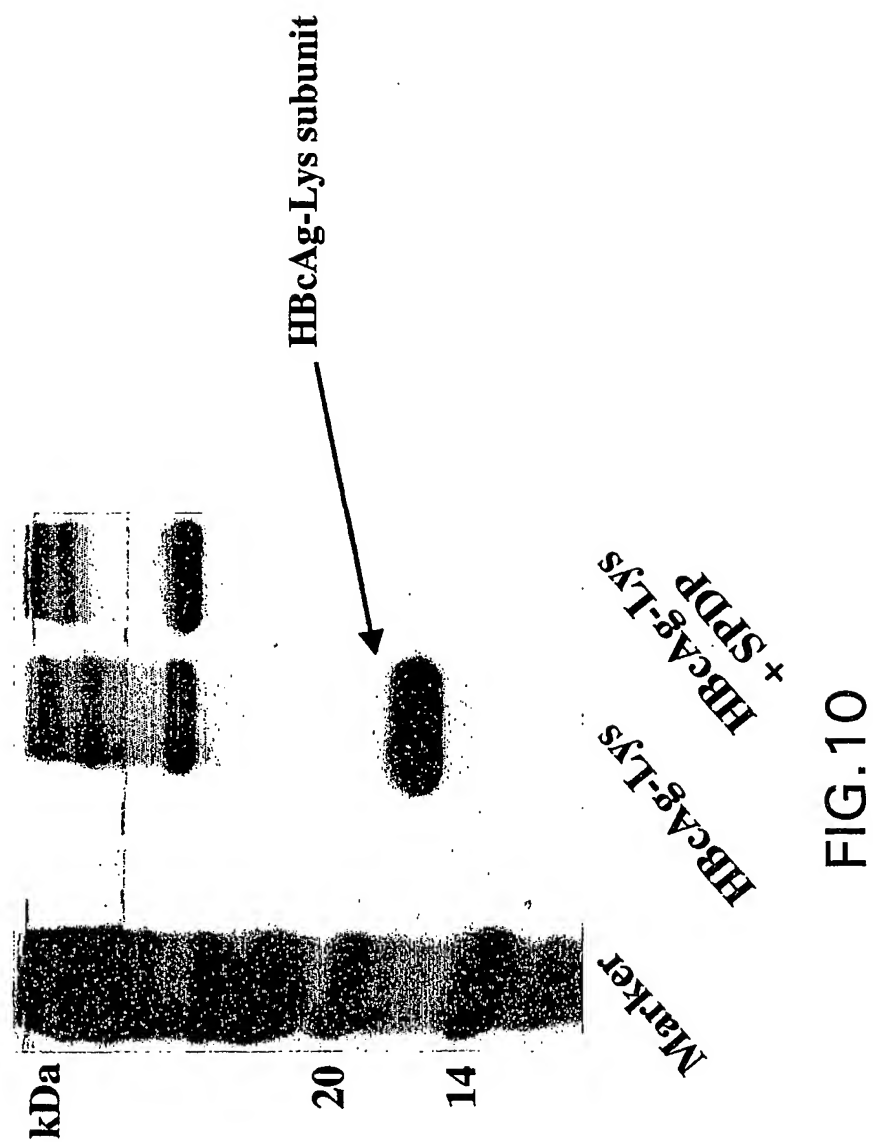
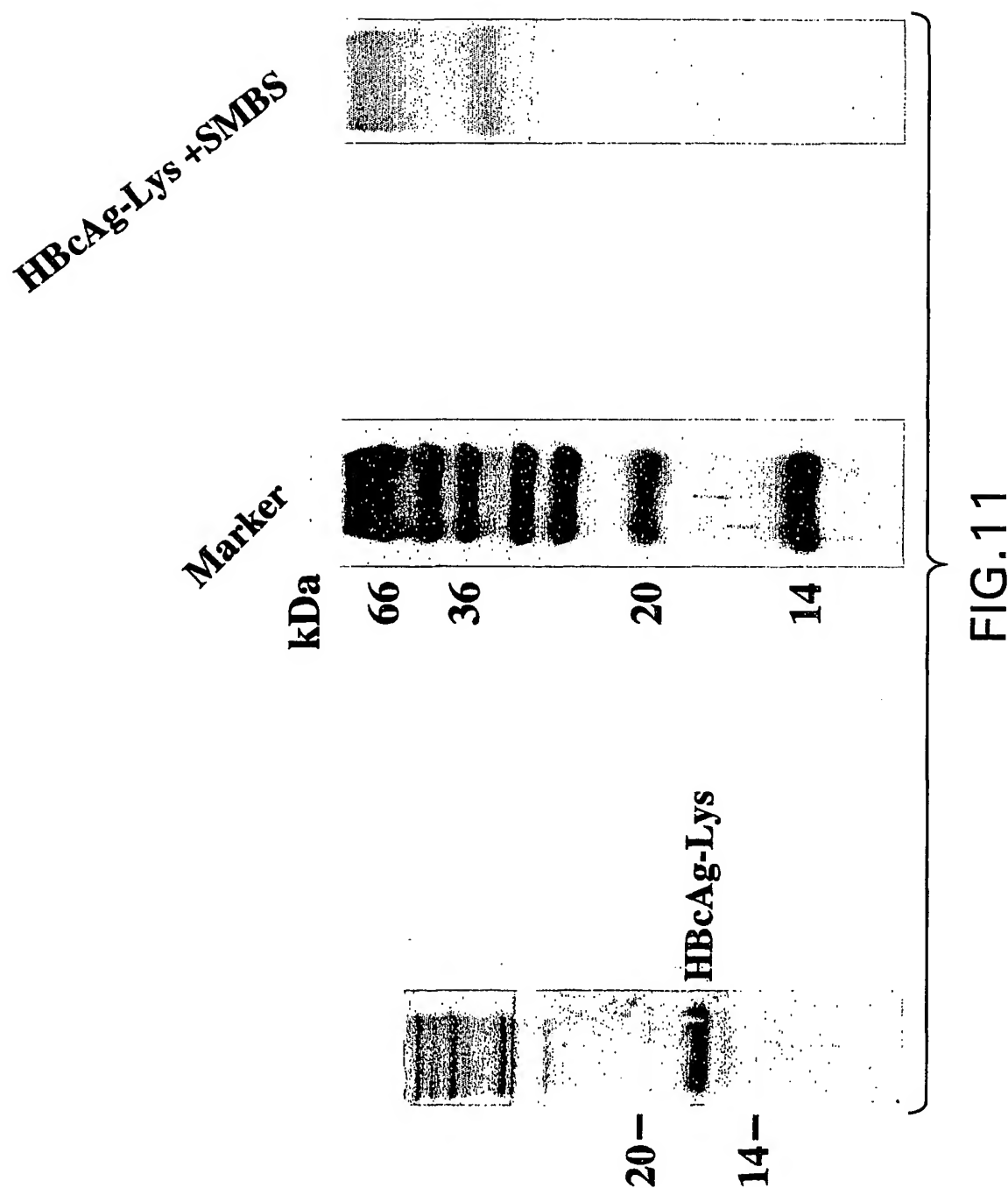


FIG.10

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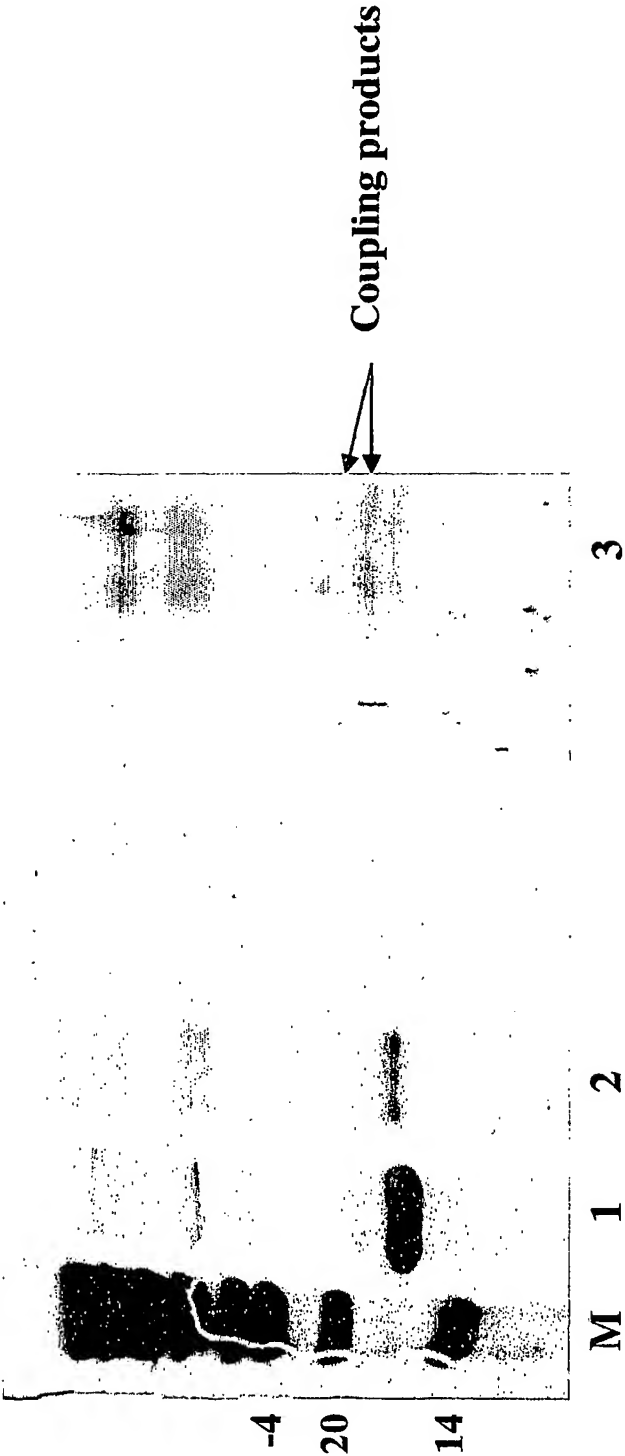


FIG.12

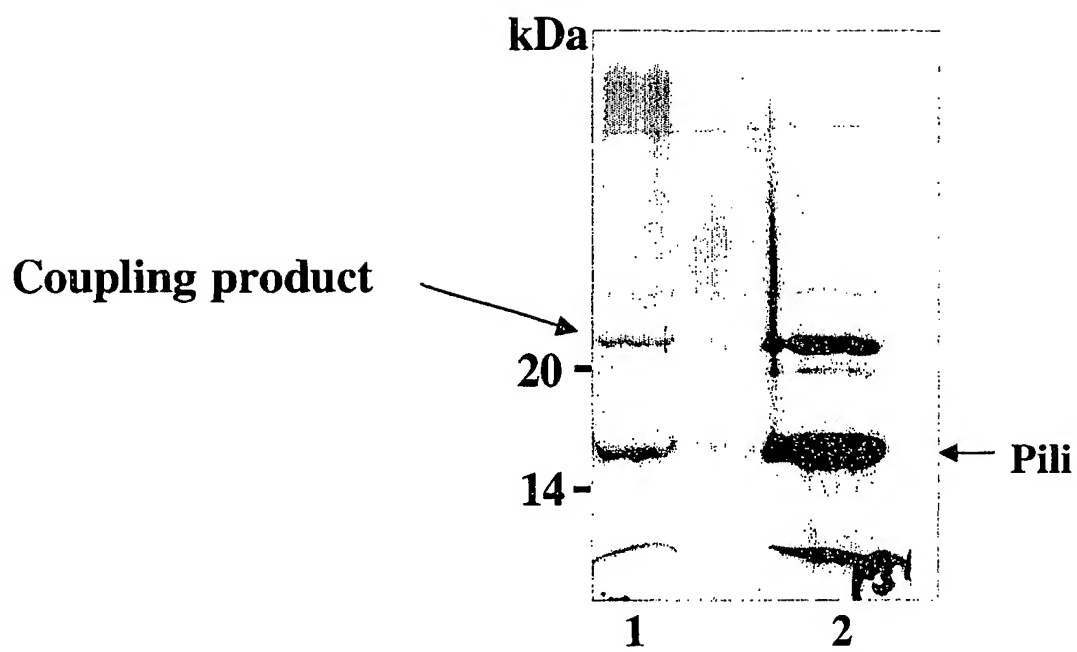


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FIG.13

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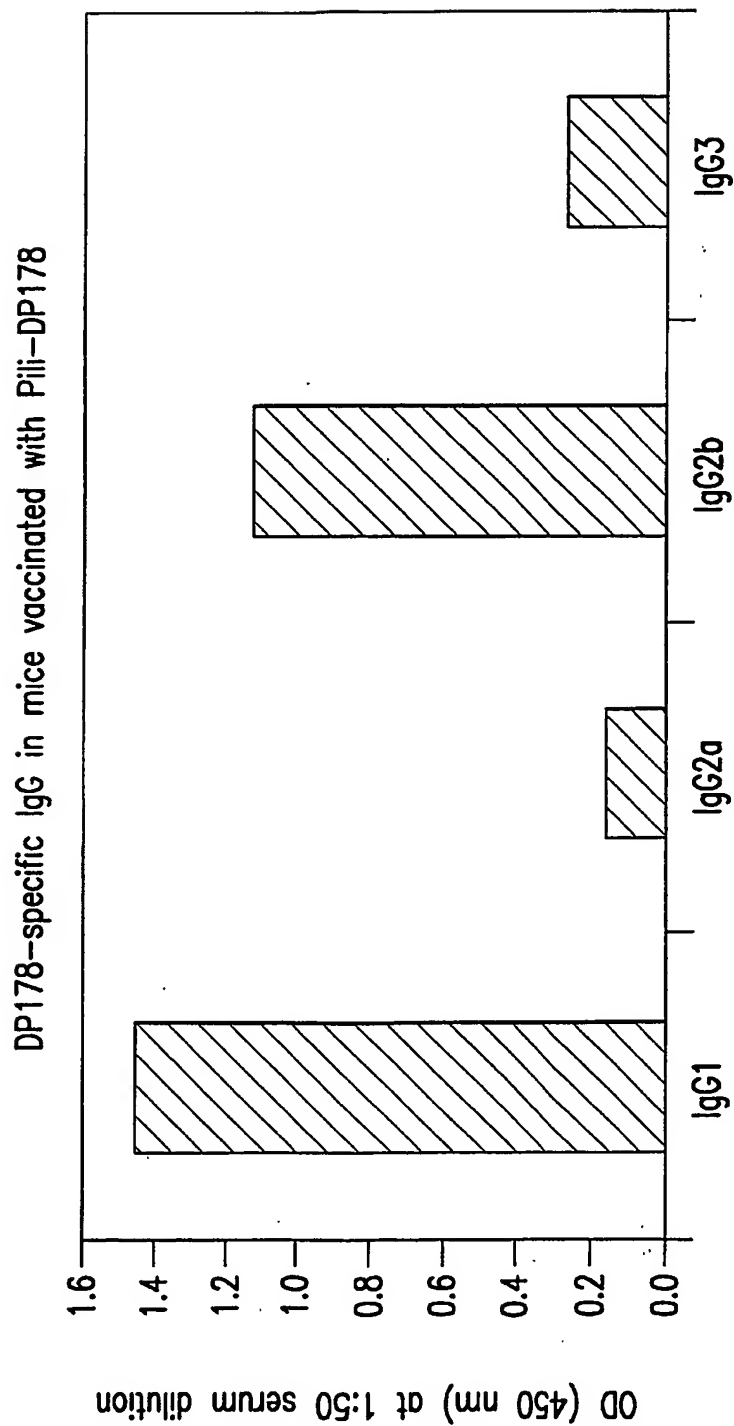


FIG. 14B

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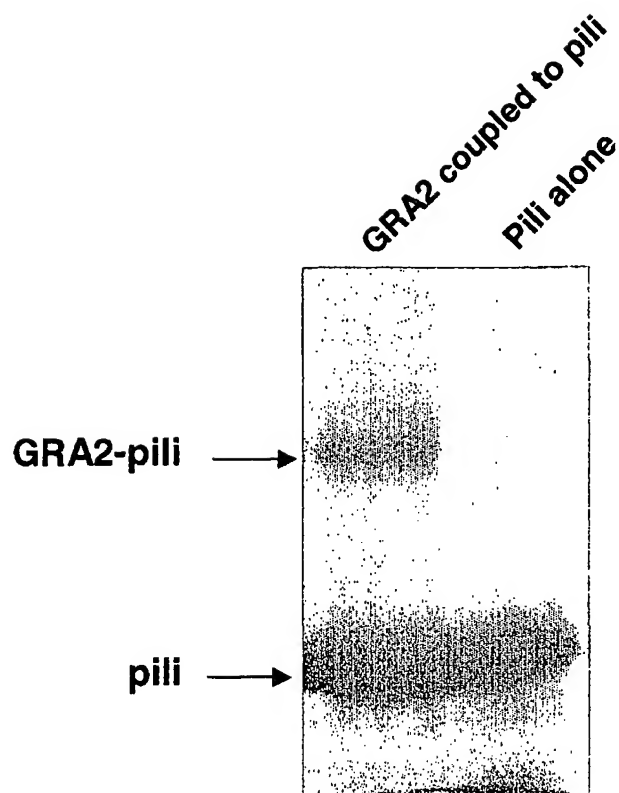


FIG.15A

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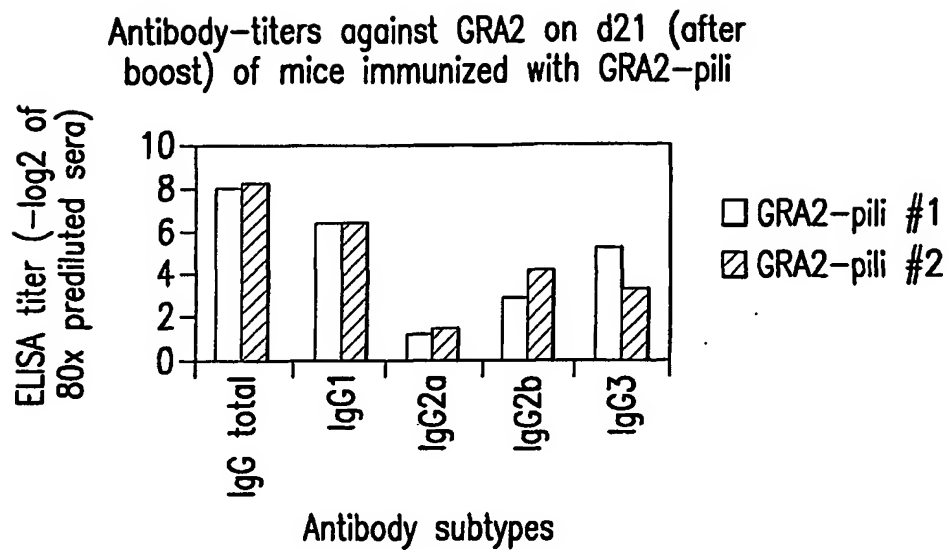


FIG.15B

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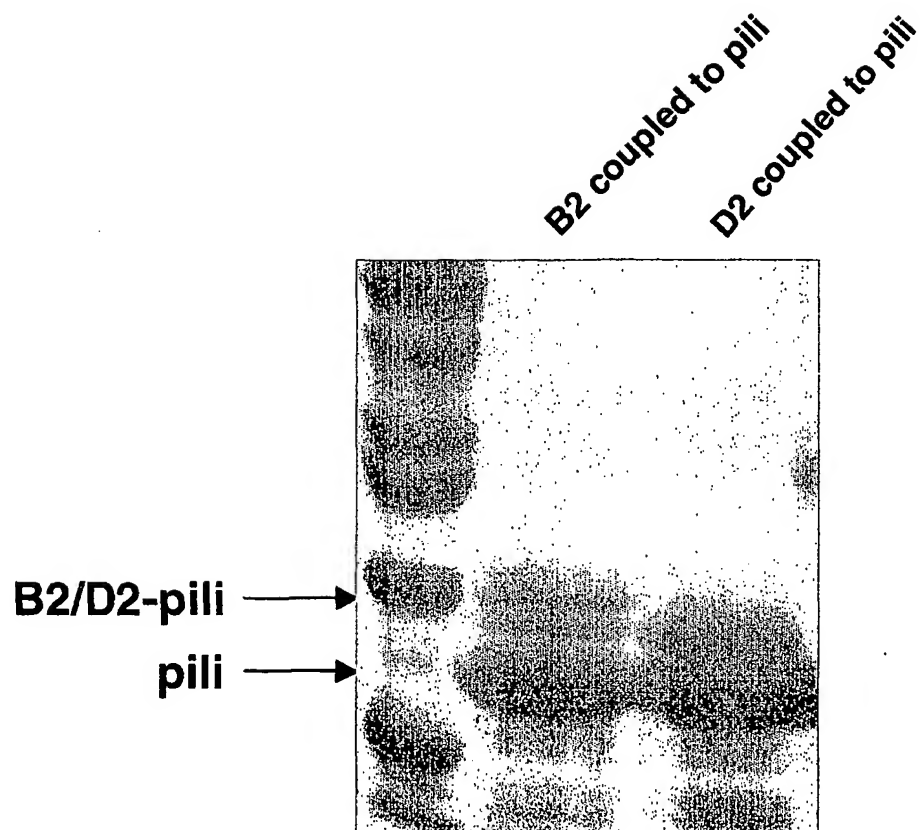


FIG.16A

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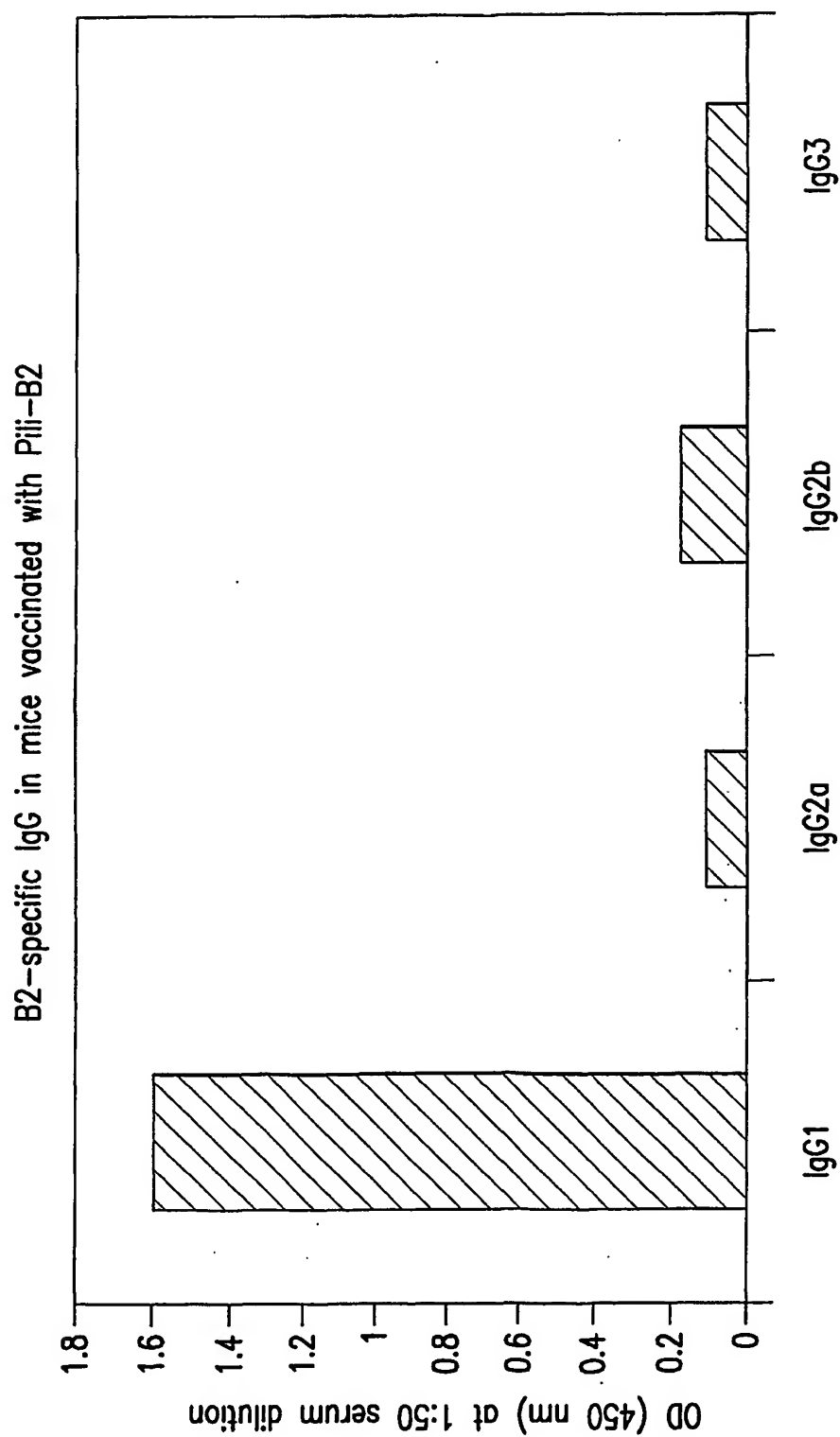


FIG.16B

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anti-TNF $\alpha$  protein IgG in sera of mice vaccinated with TNF peptide  
coupled to pili or HBc (day 20)

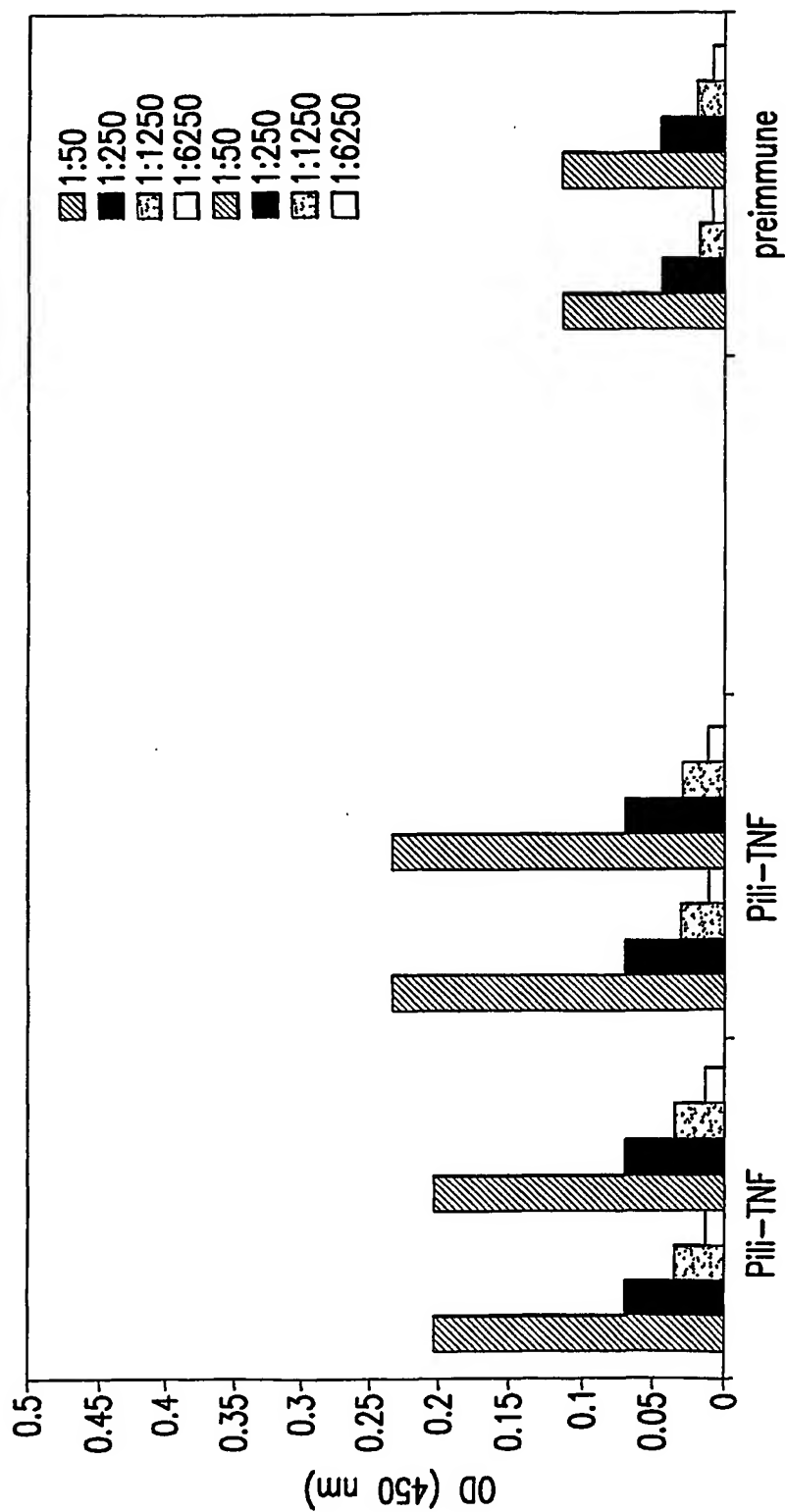


FIG.17



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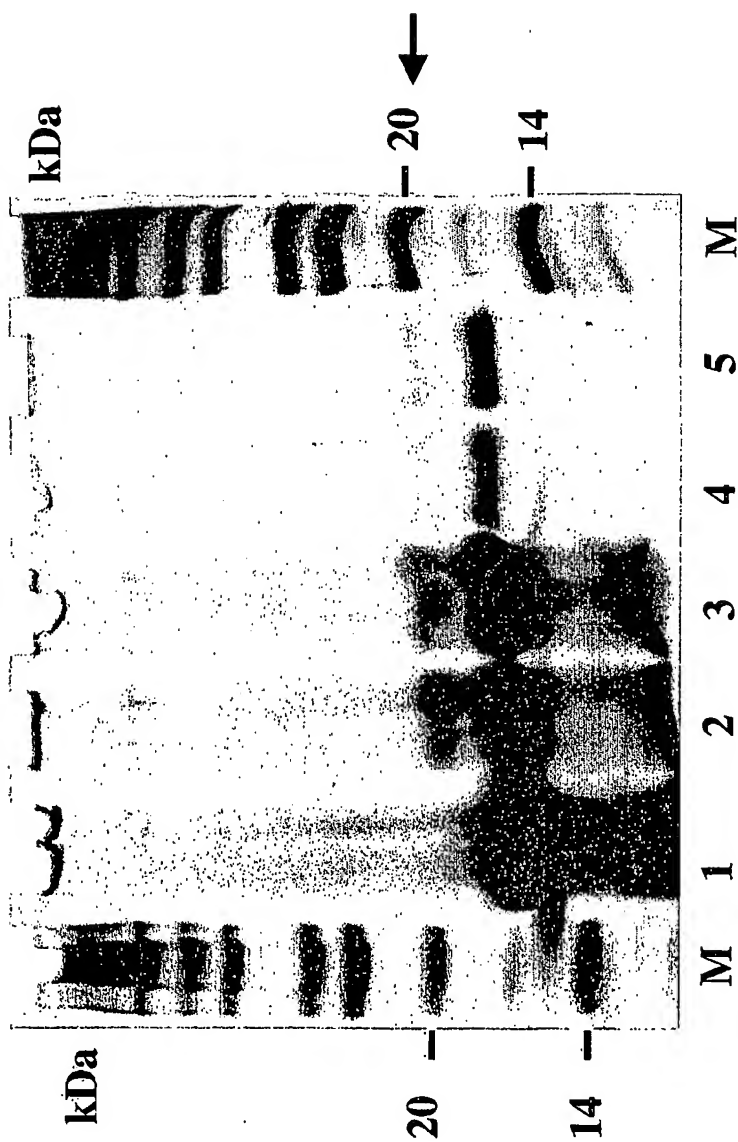


FIG.18A

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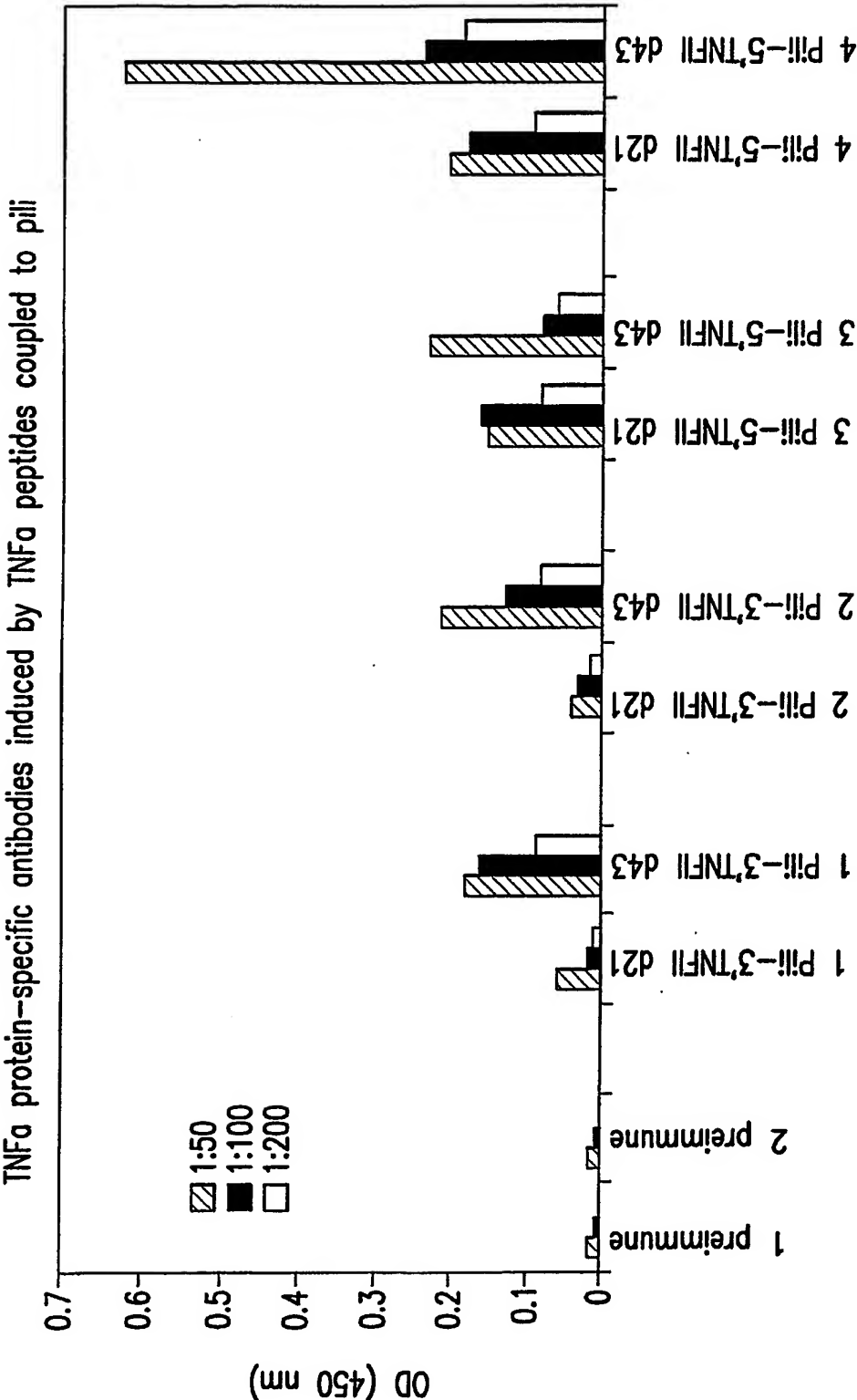


FIG.18B

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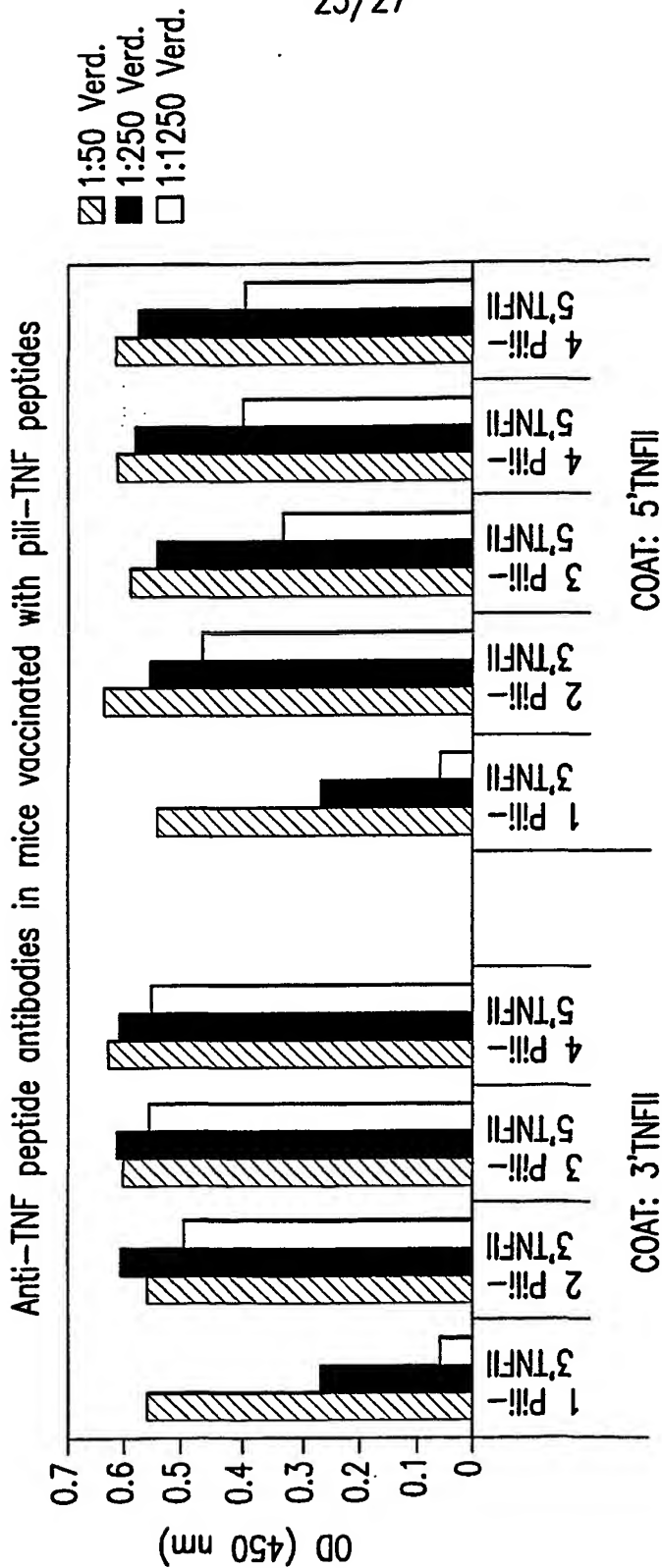


FIG.18C

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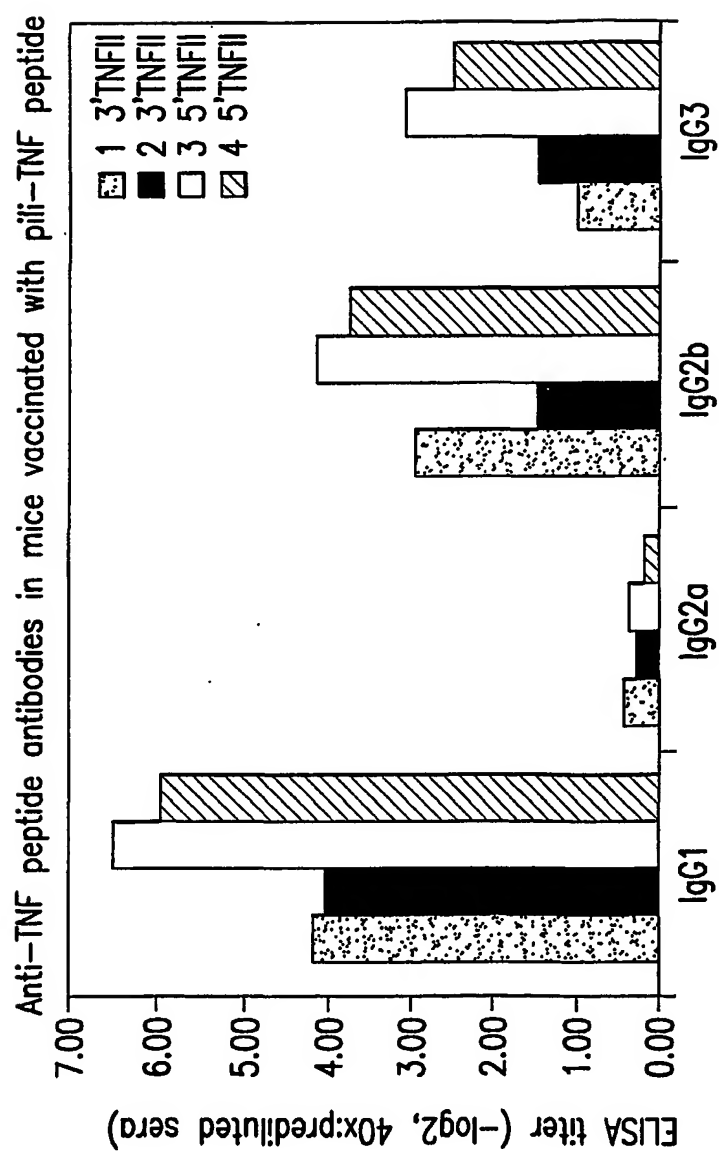


FIG.18D

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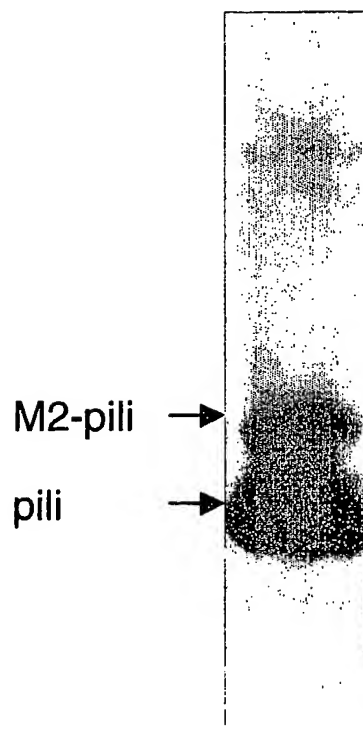


FIG.19A

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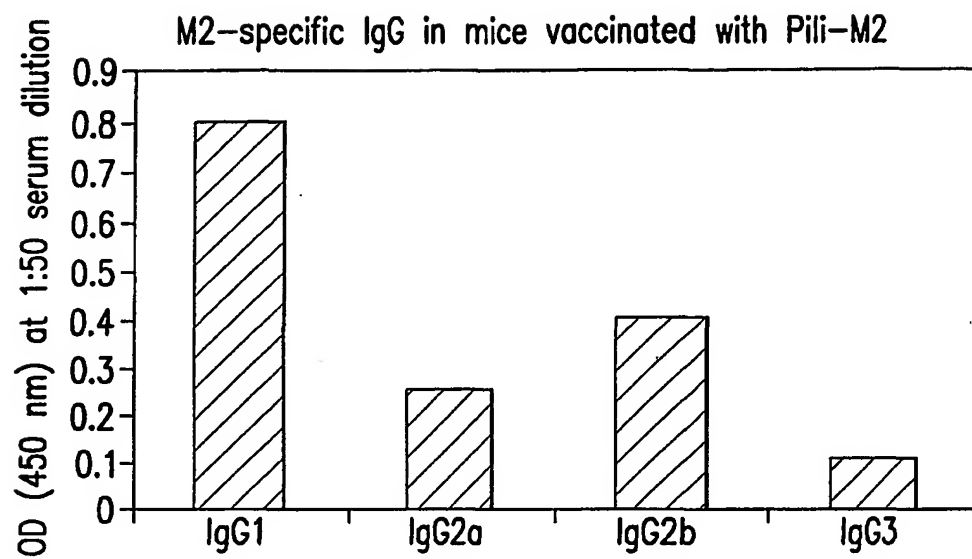


FIG.19B

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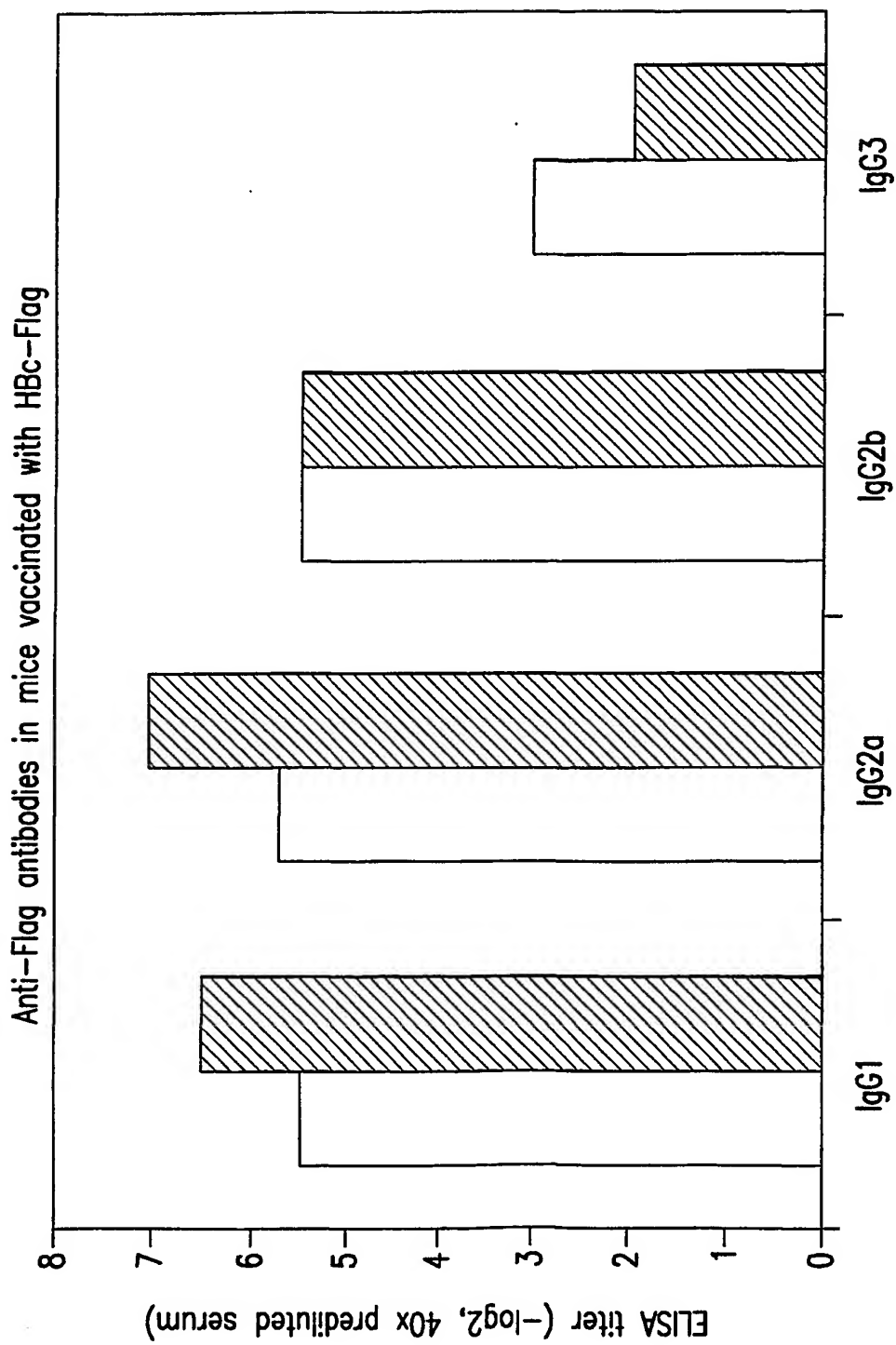


FIG.20

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## SEQUENCE LISTING

<110> Cytos Biotechnology GmbH  
Sebbel, Peter  
Dunant, Nicolas  
Bachmann, Martin  
Tissot, Alain  
Lechner, Franziska

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-2-

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&lt;400&gt; 9

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31

&lt;210&gt; 10

&lt;211&gt; 24

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&lt;223&gt; Primer

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ctgaccgaca ccctgcaggc ggaaccgac caggtggaag acgaaaaatc cgcgctgcaa 180
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gttggttgct aagctt                                     256

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                     20                    25                    30  
 Ala Asn Leu Leu Lys Glu Lys Glu Lys Leu Glu Phe Ile Leu Ala Ala  
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 His Gly Gly Cys  
                     50

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           construct

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 gca ctg gct ggt ttc gct acc gta gcg cag gcc tgc ggt ggt ctg acc 99  
 Ala Leu Ala Gly Phe Ala Thr Val Ala Gln Ala Cys Gly Gly Leu Thr  
                     15                    20                    25  
 gac acc ctg cag gcg gaa acc gac cag gtg gaa gac gaa aaa tcc gcg 147  
 Asp Thr Leu Gln Ala Glu Thr Asp Gln Val Glu Asp Glu Lys Ser Ala  
                     30                    35                    40  
 ctg caa acc gaa atc gcg aac ctg ctg aaa gaa aaa gaa aag ctg gag 195  
 Leu Gln Thr Glu Ile Ala Asn Leu Leu Lys Glu Lys Glu Lys Leu Glu  
                     45                    50                    55  
 ttc atc ctg gcg gca cac ggt ggt tgc ggt ggt tct gcg gcc gct 240  
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                   20                  25                  30  
 Thr Asp Gln Val Glu Asp Glu Lys Ser Ala Leu Gln Thr Glu Ile Ala  
           35                  40                  45  
 Asn Leu Leu Lys Glu Lys Glu Lys Leu Glu Phe Ile Leu Ala Ala His  
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 Gly Gly Leu Thr Asp Thr Leu Gln Ala Glu Thr Asp Gln Val Glu Asp  
           10                  15                  20  
 gaa aaa tcc gcg ctg caa acc gaa atc gcg aac ctg ctg aaa gaa aaa 150  
 Glu Lys Ser Ala Leu Gln Thr Glu Ile Ala Asn Leu Leu Lys Glu Lys  
           25                  30                  35  
 gaa aag ctg gag ttc atc ctg gcg gca cac ggt ggt tgc taagctt 196  
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             20                    25                    30  
 Ala Asn Leu Leu Lys Glu Lys Glu Lys Leu Glu Phe Ile Leu Ala Ala  
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             20                    25                    30  
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 20 25

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 ggtgggtctga ccgacaccct gcaggcggaa accgaccagg tggaagacga aaaatccgcg 180  
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 20 25 30  
 Ala Asn Leu Leu Lys Glu Lys Glu Lys Leu Glu Phe Ile Leu Ala Ala  
 35 40 45  
 His Gly Gly Cys  
 50

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<220>  
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        1             5             10

ctg ctc tgc ctg ccc tgg ctt caa gag ggc agc gct tgc ggt ggt ctg      96
Leu Leu Cys Leu Pro Trp Leu Gln Glu Gly Ser Ala Cys Gly Gly Leu
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acc gac acc ctg cag gcg gaa acc gac cag gtg gaa gac gaa aaa tcc      144
Thr Asp Thr Leu Gln Ala Glu Thr Asp Gln Val Glu Asp Glu Lys Ser
          35             40             45

gcg ctg caa acc gaa atc gcg aac ctg ctg aaa gaa aaa gaa aag ctg      192
Ala Leu Gln Thr Glu Ile Ala Asn Leu Leu Lys Glu Lys Glu Lys Leu
          50             55             60

gag ttc atc ctg gcg gca cac ggt ggt tgc ggt ggt tct gcg gcc gct      240
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Thr Leu Gln Ala Glu Thr Asp Gln Val Glu Asp Glu Lys Ser Ala Leu
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ggtgggaatt caggaggtaa aaaacgatgg cttgcggtgg tctgacc 47

<210> 34  
<211> 18  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 34  
gcttgcggtg gtctgacc 18

<210> 35  
<211> 27  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 35  
ccaccaagct tagcaaccac cgtgtgc 27

<210> 36  
<211> 54  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 36  
ccaccaagct tgatatcccc acaccagcg gccgcagaac caccgcaacc accg 54

<210> 37  
<211> 32  
<212> DNA  
<213> Artificial Sequence

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<220>  
<223> Primer

<400> 37  
ccaccaagct taggcctccc acaccagcg gc 32

<210> 38  
<211> 29  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 38  
ggtggaatt caggaggtaa aaaacgatg 29

<210> 39  
<211> 32  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 39  
ggtggaatt caggcctatg gctacaggct cc 32

<210> 40  
<211> 27  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 40  
ggtggaatt catggctaca ggctccc 27

<210> 41  
<211> 59  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 41  
gggtctagaa tggtacagg ctcccgagc tccctgctcc tggttttg cctgctctg 59

<210> 42  
<211> 58  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

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<400> 42  
 cgcaggcctc ggcaactgccc tcttgaagcc agggcaggca gagcaggcca aaagccag 58

<210> 43  
 <211> 402  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Modified bee  
 venom phospholipase A2

<220>  
 <221> CDS  
 <222> (1)..(402)

<400> 43  
 atc atc tac cca ggt act ctg tgg tgt ggt cac ggc aac aaa tct tct 48  
 Ile Ile Tyr Pro Gly Thr Leu Trp Cys Gly His Gly Asn Lys Ser Ser  
 1 5 10 15  
 ggt ccg aac gaa ctc ggc cgc ttt aaa cac acc gac gca tgc tgt cgc 96  
 Gly Pro Asn Glu Leu Gly Arg Phe Lys His Thr Asp Ala Cys Cys Arg  
 20 25 30  
 acc cag gac atg tgt ccg gac gtc atg tct gct ggt gaa tct aaa cac 144  
 Thr Gln Asp Met Cys Pro Asp Val Met Ser Ala Gly Glu Ser Lys His  
 35 40 45  
 ggg tta act aac acc gct tct cac acg cgt ctc agc tgc gac tgc gac 192  
 Gly Leu Thr Asn Thr Ala Ser His Thr Arg Leu Ser Cys Asp Cys Asp  
 50 55 60  
 gac aaa ttc tac gac tgc ctt aag aac tcc gcc gat acc atc tct tct 240  
 Asp Lys Phe Tyr Asp Cys Leu Lys Asn Ser Ala Asp Thr Ile Ser Ser  
 65 70 75 80  
 tac ttc gtt ggt aaa atg tat ttc aac ctg atc gat acc aaa tgt tac 288  
 Tyr Phe Val Gly Lys Met Tyr Phe Asn Leu Ile Asp Thr Lys Cys Tyr  
 85 90 95  
 aaa ctg gaa cac ccg gta acc ggc tgc ggc gaa cgt acc gaa ggt cgc 336  
 Lys Leu Glu His Pro Val Thr Gly Cys Gly Glu Arg Thr Glu Gly Arg  
 100 105 110  
 tgc ctg cac tac acc gtt gac aaa tct aaa ccg aaa gtt tac cag tgg 384  
 Cys Leu His Tyr Thr Val Asp Lys Ser Lys Pro Lys Val Tyr Gln Trp  
 115 120 125  
 ttc gac ctg cgc aaa tac 402  
 Phe Asp Leu Arg Lys Tyr  
 130

<210> 44  
 <211> 134  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Modified bee  
 venom phospholipase A2

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<400> 44  
 Ile Ile Tyr Pro Gly Thr Leu Trp Cys Gly His Gly Asn Lys Ser Ser  
 1 5 10 15  
 Gly Pro Asn Glu Leu Gly Arg Phe Lys His Thr Asp Ala Cys Cys Arg  
 20 25 30  
 Thr Gln Asp Met Cys Pro Asp Val Met Ser Ala Gly Glu Ser Lys His  
 35 40 45  
 Gly Leu Thr Asn Thr Ala Ser His Thr Arg Leu Ser Cys Asp Cys Asp  
 50 55 60  
 Asp Lys Phe Tyr Asp Cys Leu Lys Asn Ser Ala Asp Thr Ile Ser Ser  
 65 70 75 80  
 Tyr Phe Val Gly Lys Met Tyr Phe Asn Leu Ile Asp Thr Lys Cys Tyr  
 85 90 95  
 Lys Leu Glu His Pro Val Thr Gly Cys Gly Glu Arg Thr Glu Gly Arg  
 100 105 110  
 Cys Leu His Tyr Thr Val Asp Lys Ser Lys Pro Lys Val Tyr Gln Trp  
 115 120 125  
 Phe Asp Leu Arg Lys Tyr  
 130

<210> 45  
 <211> 19  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Primer

<400> 45  
 ccatcatcta cccaggtac

19

<210> 46  
 <211> 34  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Primer

<400> 46  
 cccacaccca gcggccgcgt atttgccgag gtcg

34

<210> 47  
 <211> 36  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Primer

<400> 47

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cggtggttct gcggccgcta tcattctaccc aggtac 36

<210> 48  
<211> 19  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 48  
ttagtatttg cgagggtcg 19

<210> 49  
<211> 18  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 49  
ccggctccat cgggtgcag 18

<210> 50  
<211> 36  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 50  
accaccagaa gcggccgcag gggaaacaca tctgcc 36

<210> 51  
<211> 35  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 51  
cggtggttct gcggccgctg gctccatcgg tgcag 35

<210> 52  
<211> 21  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 52  
ttaaggggaa acacatctgc c 21

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<210> 53  
<211> 33  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 53  
actagtctag aatgagagtg aaggagaaat atc 33

<210> 54  
<211> 42  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 54  
tagcatgcta gcaccgaatt tatctaattc caataattct tg 42

<210> 55  
<211> 51  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 55  
gtagcaccca ccaaggcaaa gctgaaagct acccagctcg agaaactggc a 51

<210> 56  
<211> 48  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 56  
caaagctcct attcccactg ccagtttctc gagctgggta gctttcag 48

<210> 57  
<211> 36  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 57  
ttcgggtgcta gcggtggctg cggtggtctg accgac 36

<210> 58  
<211> 37  
<212> DNA

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&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Primer

&lt;400&gt; 58

gatgctgggc ccttaaccgc aaccaccgtg tgccgcc

37

&lt;210&gt; 59

&lt;211&gt; 46

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> JUN amino acid  
sequence

&lt;400&gt; 59

Cys Gly Gly Arg Ile Ala Arg Leu Glu Glu Lys Val Lys Thr Leu Lys  
1 5 10 15Ala Gln Asn Ser Glu Leu Ala Ser Thr Ala Asn Met Leu Arg Glu Gln  
20 25 30Val Ala Gln Leu Lys Gln Lys Val Met Asn His Val Gly Cys  
35 40 45

&lt;210&gt; 60

&lt;211&gt; 46

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> FOS amino  
acid sequence

&lt;400&gt; 60

Cys Gly Gly Leu Thr Asp Thr Leu Gln Ala Glu Thr Asp Gln Val Glu  
1 5 10 15Asp Glu Lys Ser Ala Leu Gln Thr Glu Ile Ala Asn Leu Leu Lys Glu  
20 25 30Lys Glu Lys Leu Glu Phe Ile Leu Ala Ala His Gly Gly Cys  
35 40 45

&lt;210&gt; 61

&lt;211&gt; 33

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Primer

&lt;400&gt; 61

ccggaattca tgtgcggtgg tcggatcgcc cgg

33

&lt;210&gt; 62

&lt;211&gt; 39

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<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 62  
gtcgcctaccc gcggctccgc aaccaacgtg gttcatgac 39

<210> 63  
<211> 50  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 63  
gttggttgcg gagccgcggg tagcgacatt gacccttata aagaatttgg 50

<210> 64  
<211> 38  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 64  
cgcgctcccaa gcttctacgg aagcgttgat aggatagg 38

<210> 65  
<211> 33  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 65  
ctagccgcgg gttgcggtgg tcggatcgcc cgg 33

<210> 66  
<211> 38  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 66  
cgcgctcccaa gcttttagca accaacgtgg ttcattgac 38

<210> 67  
<211> 31  
<212> DNA  
<213> Artificial Sequence



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&lt;220&gt;

&lt;223&gt; Primer

&lt;400&gt; 67

ccggaattca tggacattga cccttataaa g

31

&lt;210&gt; 68

&lt;211&gt; 45

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Primer

&lt;400&gt; 68

ccgaccaccg caaccgcgg ctagcggaag cggtgatagg atagg

45

&lt;210&gt; 69

&lt;211&gt; 47

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Primer

&lt;400&gt; 69

ctaattgatc cggtaggggc tgcggtggc ggatcgccg gctcgag

47

&lt;210&gt; 70

&lt;211&gt; 39

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Primer

&lt;400&gt; 70

gtcgctaccc gcggctccgc aaccaacgtg gttcatgac

39

&lt;210&gt; 71

&lt;211&gt; 31

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Primer

&lt;400&gt; 71

ccggaattca tggacattga cccttataaa g

31

&lt;210&gt; 72

&lt;211&gt; 48

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Primer

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<400> 72  
ccgaccaccg cagccccac cggatccatt agtaccacc caggtagc 48

<210> 73  
<211> 45  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 73  
gttggttgcg gagccgcggg tagcgaccta gtagtcagtt atgtc 45

<210> 74  
<211> 38  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 74  
cgcgccccaa gcttctacgg aagcgttgat aggatagg 38

<210> 75  
<211> 33  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 75  
ctagccgcgg gttgcggtgg tcggatcgcc cgg 33

<210> 76  
<211> 38  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 76  
cgcgccccaa gcttttagca accaacgtgg ttcattgac 38

<210> 77  
<211> 30  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 77  
ccggaattca tggccacact ttttaaggagc 30

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<210> 78  
<211> 38  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 78  
cgcggtcccaa gcttttagca accaacgtgg ttcattgac 38

<210> 79  
<211> 31  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 79  
ccggaattca tggacattga cccttataaa g 31

<210> 80  
<211> 51  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 80  
cctagagcca cctttgccac catcttctaa attagtagcc acccaggtag c 51

<210> 81  
<211> 48  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 81  
gaagatggtg gcaaagggtg ctctagggac ctagtagtca gttatgtc 48

<210> 82  
<211> 38  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Primer

<400> 82  
cgcggtcccaa gcttctaaac aacagtagtc tccggaag 38

<210> 83  
<211> 36  
<212> DNA

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&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Primer

&lt;400&gt; 83

gccgaattcc tagcagctag caccgaattt atctaa

36

&lt;210&gt; 84

&lt;211&gt; 33

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Primer

&lt;400&gt; 84

ggttaagtcg acatgagagt gaaggagaaa tat

33

&lt;210&gt; 85

&lt;211&gt; 30

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Primer

&lt;400&gt; 85

taaccgaatt caggaggtaa aaagatatgg

30

&lt;210&gt; 86

&lt;211&gt; 35

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Primer

&lt;400&gt; 86

gaagtaaagc ttttaaccac cgcaaccacc agaag

35

&lt;210&gt; 87

&lt;211&gt; 33

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Primer

&lt;400&gt; 87

tcgaatgggc cctcatcttc gtgtgctagt cag

33

&lt;210&gt; 88

&lt;211&gt; 4

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

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<223> Fos fusion  
construct

<400> 88  
Glu Phe Arg Arg  
1

<210> 89  
<211> 183  
<212> PRT  
<213> Hepatitis B virus

<400> 89  
Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
1 5 10 15  
Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
20 25 30  
Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys  
35 40 45  
Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu  
50 55 60  
Leu Met Thr Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Ile  
65 70 75 80  
Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys  
85 90 95  
Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
100 105 110  
Glu Thr Val Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
115 120 125  
Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
130 135 140  
Glu Thr Thr Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr  
145 150 155 160  
Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser  
165 170 175  
Gln Ser Arg Gly Ser Gln Cys  
180

<210> 90  
<211> 183  
<212> PRT  
<213> Hepatitis B virus

<400> 90  
Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
1 5 10 15  
Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
20 25 30

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Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys  
                   35                  40                  45  
 Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu  
           50                  55                  60  
 Leu Met Thr Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Thr  
   65                  70                  75                  80  
 Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys  
                   85                  90                  95  
 Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
                  100                 105                 110  
 Glu Thr Val Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
          115                 120                 125  
 Pro Pro Ala Tyr Arg Pro Thr Asn Ala Pro Ile Leu Ser Thr Leu Pro  
   130                 135                 140  
 Glu Thr Cys Val Ile Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr  
  145                 150                 155                 160  
 Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser  
          165                 170                 175  
 Gln Ser Arg Gly Ser Gln Cys  
                  180

<210> 91  
 <211> 212  
 <212> PRT  
 <213> Hepatitis B virus

<400> 91  
 Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
   1                  5                 10                 15  
 Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
          20                 25                 30  
 Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
          35                 40                 45  
 Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
   50                 55                 60  
 Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
  65                 70                 75                 80  
 His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
          85                 90                 95  
 Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Ile Ser Arg Asp  
          100                 105                 110  
 Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
  115                 120                 125  
 Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
  130                 135                 140

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Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
195 200 205

Glu Ser Gln Cys  
210

<210> 92

<211> 212

<212> PRT

<213> Hepatitis B virus

<400> 92

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Asn Ala Ser  
50 55 60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
85 90 95

Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Ile Ser Arg Asp  
100 105 110

Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
195 200 205

Glu Ser Gln Cys  
210

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<210> 93  
 <211> 183  
 <212> PRT  
 <213> Hepatitis B virus

<400> 93  
 Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
   1                          5                          10                          15  
 Ser Phe Leu Pro Thr Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
                           20                          25                          30  
 Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys  
                           35                          40                          45  
 Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu  
                           50                          55                          60  
 Leu Met Thr Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala  
   65                          70                          75                          80  
 Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys  
                           85                          90                          95  
 Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
                           100                          105                          110  
 Glu Thr Val Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
                           115                          120                          125  
 Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
   130                          135                          140  
 Glu Thr Cys Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr  
   145                          150                          155                          160  
 Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser  
                           165                          170                          175  
 Gln Ser Arg Glu Ser Gln Cys  
                           180

<210> 94  
 <211> 212  
 <212> PRT  
 <213> Hepatitis B virus

<400> 94  
 Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
   1                          5                          10                          15  
 Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
                           20                          25                          30  
 Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
                           35                          40                          45  
 Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
   50                          55                          60  
 Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His



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65		70		75		80
His Thr Ala Leu Arg	Gln Ala Ile Leu Cys Trp Gly Asp Leu Met Thr					
	85		90		95	
Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Val Ser Arg Asp			105		110	
	100					
Leu Val Val Ser Tyr Val Asn Thr Asn Val Gly Leu Lys Phe Arg Gln			120		125	
	115					
Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val			135		140	
	130					
Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala			150		155	160
	145					
Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr			165		170	175
Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro			180		185	190
Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg			195		200	205
Glu Ser Gln Cys						
	210					

<210> 95  
 <211> 212  
 <212> PRT  
 <213> Hepatitis B virus

<400> 95
Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr
1 5 10 15
Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Asp Met Asp Ile
20 25 30
Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu
35 40 45
Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser
50 55 60
Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His
65 70 75 80
His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp Leu Met Thr
85 90 95
Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Val Ser Arg Asp
100 105 110
Leu Val Val Ser Tyr Val Asn Thr Asn Val Gly Leu Lys Phe Arg Gln
115 120 125
Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val
130 135 140

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Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
195 200 205

Glu Ser Gln Cys  
210

<210> 96

<211> 212

<212> PRT

<213> Hepatitis B virus

<400> 96

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
50 55 60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro Gln  
65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
85 90 95

Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Ile Ser Arg Asp  
100 105 110

Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
195 200 205

Glu Ser Gln Cys  
210

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<210> 97  
 <211> 212  
 <212> PRT  
 <213> Hepatitis B virus

<400> 97  
 Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
   1                  5                  10                  15  
 Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
                   20                  25                  30  
 Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
                   35                  40                  45  
 Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
                   50                  55                  60  
 Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
   65                  70                  75                  80  
 His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
                   85                  90                  95  
 Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp  
                   100                  105                  110  
 Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
                   115                  120                  125  
 Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
   130                  135                  140  
 Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145                  150                  155                  160  
 Tyr Lys Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
                   165                  170                  175  
 Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
                   180                  185                  190  
 Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
   195                  200                  205  
 Gly Ser Gln Cys  
   210

<210> 98  
 <211> 183  
 <212> PRT  
 <213> Hepatitis B virus

<400> 98  
 Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
   1                  5                  10                  15  
 Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
                   20                  25                  30  
 Thr Ala Ser Ala Leu Phe Arg Asp Ala Leu Glu Ser Pro Glu His Cys

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35                      40                      45  
 Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu  
     50                      55                      60  
 Leu Met Thr Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Ala  
     65                      70                      75                      80  
 Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys  
                     85                      90                      95  
 Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
                     100                      105                      110  
 Asp Thr Val Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
                     115                      120                      125  
 Pro Pro Ala Tyr Arg Pro Ser Asn Ala Pro Ile Leu Ser Thr Leu Pro  
                     130                      135                      140  
 Glu Thr Cys Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr  
     145                      150                      155                      160  
 Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser  
                     165                      170                      175  
 Gln Ser Arg Glu Ser Gln Cys  
                     180

<210> 99  
 <211> 183  
 <212> PRT  
 <213> Hepatitis B virus

<400> 99  
 Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
     1                      5                      10                      15  
 Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
                     20                      25                      30  
 Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys  
                     35                      40                      45  
 Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu  
     50                      55                      60  
 Leu Met Thr Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala  
     65                      70                      75                      80  
 Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys  
                     85                      90                      95  
 Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
                     100                      105                      110  
 Glu Thr Val Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
                     115                      120                      125  
 Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
                     130                      135                      140

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Glu Thr Thr Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr  
145 150 155 160

Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser  
165 170 175

Gln Ser Arg Glu Ser Gln Cys  
180

&lt;210&gt; 100

&lt;211&gt; 212

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 100

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
50 55 60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
65 70 75 80

His Thr Ala Leu Arg His Ala Ile Leu Cys Trp Gly Asp Leu Arg Thr  
85 90 95

Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Ile Ser Arg Asp  
100 105 110

Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
195 200 205

Glu Ser Gln Cys  
210

&lt;210&gt; 101

&lt;211&gt; 212

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

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&lt;400&gt; 101

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
 1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Asp Met Asp Ile  
 20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
 35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
 50 55 60

Ala Leu Phe Arg Asp Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
 65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
 85 90 95

Leu Ala Thr Trp Val Gly Ala Asn Leu Glu Asp Pro Ala Ser Arg Asp  
 100 105 110

Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
 115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Gln Ala  
 145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Cys  
 165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
 180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
 195 200 205

Glu Ser Gln Cys  
 210

&lt;210&gt; 102

&lt;211&gt; 183

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> synthetic  
 human Hepatitis B construct

&lt;400&gt; 102

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
 1 5 10 15

Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
 20 25 30

Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys  
 35 40 45

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Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu  
 50 55 60  
 Leu Met Thr Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala  
 65 70 75 80  
 Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys  
 85 90 95  
 Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
 100 105 110  
 Glu Thr Val Leu Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
 115 120 125  
 Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
 130 135 140  
 Glu Thr Thr Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr  
 145 150 155 160  
 Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser  
 165 170 175  
 Gln Ser Arg Glu Ser Gln Cys  
 180

<210> 103  
 <211> 212  
 <212> PRT  
 <213> Hepatitis B virus

<400> 103  
 Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
 1 5 10 15  
 Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
 20 25 30  
 Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
 35 40 45  
 Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
 50 55 60  
 Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
 65 70 75 80  
 His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp Leu Met Ser  
 85 90 95  
 Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ile Ser Arg Asp  
 100 105 110  
 Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
 115 120 125  
 Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130 135 140  
 Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145 150 155 160

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Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
195 200 205

Glu Ser Gln Cys  
210

&lt;210&gt; 104

&lt;211&gt; 183

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 104

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
1 5 10 15

Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
20 25 30

Thr Ala Ser Ala Leu Tyr Arg Asp Ala Leu Glu Ser Pro Glu His Cys  
35 40 45

Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu  
50 55 60

Leu Met Thr Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala  
65 70 75 80

Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys  
85 90 95

Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
100 105 110

Glu Thr Val Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
115 120 125

Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
130 135 140

Glu Thr Thr Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr  
145 150 155 160

Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser  
165 170 175

Gln Ser Arg Glu Ser Gln Cys  
180

&lt;210&gt; 105

&lt;211&gt; 183

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 105

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu



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1	5	10	15
Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp	20	25	30
Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys	35	40	45
Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp	50	55	60
Leu Met Thr Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala	65	70	75
Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys	85	90	95
Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg	100	105	110
Glu Thr Val Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr	115	120	125
Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro	130	135	140
Glu Thr Thr Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr	145	150	155
Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser	165	170	175
Gln Ser Arg Glu Ser Gln Cys	180		

&lt;210&gt; 106

&lt;211&gt; 183

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 106

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu	1	5	10	15
Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp	20	25	30	
Thr Ala Ser Ala Leu Tyr Arg Asp Ala Leu Glu Ser Pro Glu His Cys	35	40	45	
Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu	50	55	60	
Leu Met Thr Leu Ala Thr Trp Val Gly Ala Asn Leu Glu Asp Pro Ala	65	70	75	80
Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys	85	90	95	
Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg	100	105	110	

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Glu Thr Val Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
 115 120 125

Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
 130 135 140

Glu Thr Thr Val Val Arg Arg Arg Gly Arg Thr Pro Arg Arg Arg Thr  
 145 150 155 160

Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser  
 165 170 175

Gln Ser Arg Glu Ser Gln Cys  
 180

<210> 107  
 <211> 212  
 <212> PRT  
 <213> Hepatitis B virus

<400> 107  
 Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
 1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
 20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
 35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
 50 55 60

Ala Leu Tyr Arg Asp Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
 65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
 85 90 95

Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp  
 100 105 110

Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
 115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
 165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
 180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
 195 200 205

Glu Ser Gln Cys  
 210

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<210> 108  
 <211> 212  
 <212> PRT  
 <213> Hepatitis B virus

<400> 108  
 Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
   1                  5                  10                  15  
 Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
                   20                  25                  30  
 Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
                   35                  40                  45  
 Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
           50                  55                  60  
 Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
   65                  70                  75                  80  
 His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp Leu Met Thr  
                   85                  90                  95  
 Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp  
                   100                  105                  110  
 Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
           115                  120                  125  
 Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
   130                  135                  140  
 Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
   145                  150                  155                  160  
 Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
                   165                  170                  175  
 Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
           180                  185                  190  
 Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
   195                  200                  205  
 Glu Ser Gln Cys  
   210

<210> 109  
 <211> 212  
 <212> PRT  
 <213> Hepatitis B virus

<400> 109  
 Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Thr Cys Pro Thr  
   1                  5                  10                  15  
 Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
                   20                  25                  30  
 Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu

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35                                      40                                      45  
 Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
     50                                      55                                      60  
 Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
     65                                      70                                      75                                      80  
 His Thr Ala Leu Arg Glu Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
                                     85                                      90                                      95  
 Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp  
                                     100                                      105                                      110  
 Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
                                     115                                      120                                      125  
 Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
     130                                      135                                      140  
 Ile Glu Tyr Leu Val Ala Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
     145                                      150                                      155                                      160  
 Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
                                     165                                      170                                      175  
 Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
                                     180                                      185                                      190  
 Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
                                     195                                      200                                      205  
 Glu Ser Gln Cys  
     210

<210> 110  
 <211> 212  
 <212> PRT  
 <213> Hepatitis B virus

<400> 110  
 Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
     1                                      5                                      10                                      15  
 Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
                                     20                                      25                                      30  
 Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
                                     35                                      40                                      45  
 Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
     50                                      55                                      60  
 Ala Leu Tyr Arg Glu Ala Phe Glu Cys Ser Glu His Cys Ser Pro His  
     65                                      70                                      75                                      80  
 His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
                                     85                                      90                                      95  
 Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Ile Ser Arg Asp  
                                     100                                      105                                      110

[illegible]

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<400> 111
Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr
  1          5          10          15
Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Xaa Asp Met Asp Ile
          20          25          30
Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu
          35          40          45
Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser
          50          55          60
Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His
  65          70          75          80
His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp Leu Ile Thr
          85          90          95
Leu Ser Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Thr Ser Arg Asp
          100          105          110
Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln
          115          120          125
Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val
          130          135          140
Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala
  145          150          155          160

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[illegible][illegible]

<210> 113

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&lt;211&gt; 212

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 113

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
 1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
 20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
 35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
 50 55 60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
 65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
 85 90 95

Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp  
 100 105 110

Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
 115 120 125

Leu Leu Trp Phe His Ile Cys Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
 165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
 180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
 195 200 205

Glu Ser Gln Cys  
 210

&lt;210&gt; 114

&lt;211&gt; 212

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 114

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
 1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
 20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
 35 40 45

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Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
 50 55 60  
 Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
 65 70 75 80  
 His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
 85 90 95  
 Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp  
 100 105 110  
 Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
 115 120 125  
 Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130 135 140  
 Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145 150 155 160  
 Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
 165 170 175  
 Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
 180 185 190  
 Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
 195 200 205  
 Glu Pro Gln Cys  
 210

<210> 115  
 <211> 212  
 <212> PRT  
 <213> Hepatitis B virus

<400> 115  
 Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
 1 5 10 15  
 Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
 20 25 30  
 Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
 35 40 45  
 Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Ser Thr Ala Ser  
 50 55 60  
 Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
 65 70 75 80  
 His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
 85 90 95  
 Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp  
 100 105 110  
 Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
 115 120 125



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Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130 135 140  
 Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145 150 155 160  
 Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
 165 170 175  
 Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
 180 185 190  
 Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
 195 200 205  
 Glu Ser Gln Cys  
 210

&lt;210&gt; 116

&lt;211&gt; 212

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 116

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
 1 5 10 15  
 Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
 20 25 30  
 Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
 35 40 45  
 Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
 50 55 60  
 Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
 65 70 75 80  
 His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
 85 90 95  
 Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp  
 100 105 110  
 Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
 115 120 125  
 Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130 135 140  
 Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145 150 155 160  
 Tyr Arg Pro Pro Asn Ala Pro Ile Leu Leu Thr Leu Pro Glu Thr Thr  
 165 170 175  
 Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
 180 185 190  
 Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
 195 200 205

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Glu Ser Gln Cys  
210

<210> 117  
<211> 212  
<212> PRT  
<213> Hepatitis B virus

<400> 117  
Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
1 5 10 15  
Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
20 25 30  
Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
35 40 45  
Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
50 55 60  
Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
65 70 75 80  
His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp Leu Met Thr  
85 90 95  
Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp  
100 105 110  
Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Lys Gln  
115 120 125  
Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
130 135 140  
Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
145 150 155 160  
Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
165 170 175  
Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
180 185 190  
Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
195 200 205  
Glu Ser Gln Cys  
210

<210> 118  
<211> 212  
<212> PRT  
<213> Hepatitis B virus

<400> 118  
Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
1 5 10 15  
Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile

20

30

```
<210> 119
<211> 183
<212> PRT
<213> Hepatitis B virus
```

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Ser Met Glu Leu Leu  
1 5 10 15

Ser Phe Leu Pro Ser Asp Phe Tyr Pro Ser Val Arg Asp Leu Leu Asp  
20 25 30

Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys  
35 40 45

Thr Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu  
50 55 60

Leu Met Thr Leu Ala Thr Trp Val Gly Gly Asn Leu Gln Asp Pro Thr  
65 70 75 80

Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys  
85 90 95

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Phe Arg Gln Leu Leu Trp Phe His Val Ser Cys Leu Thr Phe Gly Arg  
                   100                  105                  110  
 Glu Thr Val Val Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
                   115                  120                  125  
 Pro Gln Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
                   130                  135                  140  
 Glu Thr Cys Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr  
                   145                  150                  155                  160  
 Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser  
                   165                  170                  175  
 Gln Ser Arg Glu Ser Gln Cys  
                   180

<210> 120  
 <211> 183  
 <212> PRT  
 <213> Hepatitis B virus

<400> 120  
 Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
   1                  5                  10                  15  
 Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
                   20                  25                  30  
 Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys  
                   35                  40                  45  
 Ser Pro His His Thr Ala Leu Arg His Val Phe Leu Cys Trp Gly Asp  
                   50                  55                  60  
 Leu Met Thr Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Thr  
                   65                  70                  75                  80  
 Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys  
                   85                  90                  95  
 Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
                   100                  105                  110  
 Glu Thr Val Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
                   115                  120                  125  
 Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
                   130                  135                  140  
 Glu Thr Thr Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr  
                   145                  150                  155                  160  
 Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser  
                   165                  170                  175  
 Gln Ser Arg Glu Ser Gln Cys  
                   180

<210> 121

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&lt;211&gt; 212

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 121

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
 1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
 20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
 35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
 50 55 60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
 65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp Leu Thr Thr  
 85 90 95

Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp  
 100 105 110

Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
 115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
 165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
 180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
 195 200 205

Glu Ser Gln Cys  
 210

&lt;210&gt; 122

&lt;211&gt; 212

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 122

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
 1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
 20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
 35 40 45

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Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
 50 55 60  
 Ala Leu Tyr Arg Asp Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
 65 70 75 80  
 His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr  
 85 90 95  
 Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp  
 100 105 110  
 Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
 115 120 125  
 Leu Leu Trp Phe His Ile Ser Cys Leu Ile Phe Gly Arg Glu Thr Val  
 130 135 140  
 Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145 150 155 160  
 Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
 165 170 175  
 Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
 180 185 190  
 Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
 195 200 205  
 Glu Ser Gln Cys  
 210

&lt;210&gt; 123

&lt;211&gt; 183

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 123

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
 1 5 10 15  
 Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
 20 25 30  
 Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys  
 35 40 45  
 Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp  
 50 55 60  
 Leu Met Thr Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Val  
 65 70 75 80  
 Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Val Gly Leu Lys  
 85 90 95  
 Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
 100 105 110  
 Glu Thr Val Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
 115 120 125

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Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
 130 135 140

Glu Thr Thr Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr  
 145 150 155 160

Pro Ser Pro Ala Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser  
 165 170 175

Gln Ser Arg Glu Ser Gln Cys  
 180

<210> 124  
 <211> 212  
 <212> PRT  
 <213> Hepatitis B virus

<400> 124  
 Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
 1 5 10 15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
 20 25 30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
 35 40 45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
 50 55 60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
 65 70 75 80

His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp Leu Met Asn  
 85 90 95

Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Val Ser Arg Asp  
 100 105 110

Leu Val Val Gly Tyr Val Asn Thr Thr Val Gly Leu Lys Phe Arg Gln  
 115 120 125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130 135 140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145 150 155 160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
 165 170 175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
 180 185 190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
 195 200 205

Glu Ser Gln Cys  
 210

<210> 125

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&lt;211&gt; 183

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 125

```

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu
 1           5           10           15

Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp
          20           25           30

Thr Ala Ser Ala Leu Tyr Arg Asp Ala Leu Glu Ser Pro Glu His Cys
      35           40           45

Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp
      50           55           60

Leu Met Thr Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala
 65           70           75           80

Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys
          85           90           95

Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg
      100           105           110

Glu Thr Val Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr
      115           120           125

Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro
      130           135           140

Glu Thr Thr Val Val Arg Arg Arg Gly Arg Thr Pro Arg Arg Arg Thr
      145           150           155           160

Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser
          165           170           175

Gln Ser Arg Glu Ser Gln Cys
      180

```

&lt;210&gt; 126

&lt;211&gt; 212

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 126

```

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr
 1           5           10           15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile
      20           25           30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu
      35           40           45

Pro Ser Asp Phe Phe Pro Ser Val Arg Ala Leu Leu Asp Thr Ala Ser
      50           55           60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His
      65           70           75           80

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[illegible]

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<210> 127  
<211> 212  
<212> PRT  
<213> Hepatitis B virus
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<400> 127
Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr
  1              5              10              15
Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile
      20              25              30
Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu
      35              40              45
Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser
      50              55              60
Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His
  65              70              75              80
His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp Leu Met Thr
      85              90              95
Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Thr Arg Asp
      100              105              110
Leu Val Val Ser Tyr Val Asn Thr Asn Val Gly Leu Lys Phe Arg Gln
      115              120              125
Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val
      130              135              140
Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala
  145              150              155              160

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Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
                   165                  170                  175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
                   180                  185                  190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
                   195                  200                  205

Glu Ser Gln Cys  
       210

<210> 128  
 <211> 212  
 <212> PRT  
 <213> Hepatitis B virus

<400> 128  
 Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr  
       1                  5                  10                  15

Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile  
                   20                  25                  30

Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu  
                   35                  40                  45

Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
                   50                  55                  60

Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
       65                  70                  75                  80

His Thr Ala Leu Arg Gln Arg Ile Leu Cys Trp Gly Glu Leu Met Thr  
                   85                  90                  95

Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp  
                   100                  105                  110

Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Glu  
                   115                  120                  125

Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
                   130                  135                  140

Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
       145                  150                  155                  160

Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
                   165                  170                  175

Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
                   180                  185                  190

Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Thr Arg Ser Gln Ser Arg  
                   195                  200                  205

Glu Ser Gln Cys  
       210

<210> 129

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&lt;211&gt; 212

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 129

```

Met Gln Leu Phe His Leu Cys Leu Val Ile Ser Cys Ser Cys Pro Thr
 1           5           10          15
Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile
          20           25          30
Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu
          35           40          45
Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ala
          50           55          60
Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His
          65           70          75          80
His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr
          85           90          95
Leu Ala Thr Trp Val Gly Asn Asn Leu Glu Asp Pro Ala Ser Arg Asp
          100          105          110
Leu Val Val Asn Tyr Val Asn Thr Asn Met Gly Leu Lys Ile Arg Gln
          115          120          125
Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val
          130          135          140
Leu Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala
          145          150          155          160
Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr
          165          170          175
Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro
          180          185          190
Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg
          195          200          205
Glu Ser Gln Cys
          210

```

&lt;210&gt; 130

&lt;211&gt; 212

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 130

```

Met Gln Leu Phe His Leu Cys Leu Ile Ile Ser Cys Ser Cys Pro Thr
 1           5           10          15
Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile
          20           25          30
Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu
          35           40          45

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Pro Ser Ala Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser  
 50 55 60  
 Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His  
 65 70 75 80  
 His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp Leu Met Thr  
 85 90 95  
 Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp  
 100 105 110  
 Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys Phe Arg Gln  
 115 120 125  
 Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val  
 130 135 140  
 Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala  
 145 150 155 160  
 Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr  
 165 170 175  
 Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro  
 180 185 190  
 Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg  
 195 200 205  
 Glu Ser Gln Cys  
 210

<210> 131  
 <211> 183  
 <212> PRT  
 <213> Hepatitis B virus

<400> 131  
 Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
 1 5 10 15  
 Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
 20 25 30  
 Thr Ala Ala Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys  
 35 40 45  
 Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu  
 50 55 60  
 Leu Met Thr Leu Ala Thr Trp Val Gly Asn Asn Leu Glu Asp Pro Ala  
 65 70 75 80  
 Ser Arg Asp Leu Val Val Asn Tyr Val Asn Thr Asn Met Gly Leu Lys  
 85 90 95  
 Ile Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
 100 105 110  
 Glu Thr Val Leu Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
 115 120 125

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Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
 130 135 140

Glu Thr Thr Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr  
 145 150 155 160

Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser  
 165 170 175

Gln Ser Arg Glu Ser Gln Cys  
 180

<210> 132  
 <211> 183  
 <212> PRT  
 <213> Hepatitis B virus

<400> 132  
 Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
 1 5 10 15

Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
 20 25 30

Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys  
 35 40 45

Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu  
 50 55 60

Leu Met Thr Leu Ala Thr Trp Val Gly Gly Asn Leu Glu Asp Pro Ile  
 65 70 75 80

Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys  
 85 90 95

Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
 100 105 110

Glu Thr Val Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
 115 120 125

Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
 130 135 140

Glu Thr Cys Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr  
 145 150 155 160

Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser  
 165 170 175

Gln Ser Arg Gly Ser Gln Cys  
 180

<210> 133  
 <211> 3221  
 <212> DNA  
 <213> Hepatitis B virus

<220>  
 <221> CDS  
 <222> (1901)..(2458)

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&lt;400&gt; 133

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tgggtggctcc agttcaggaa cagtaaacc tgctccgaat attgcctctc acatctcgtc 120  
aatctccgcg aggactgggg accctgtgac gaacatggag aacatcacat caggattcct 180  
aggacccctg ctctgtgttac aggcgggggt tttattgttg acaagaatcc tcacaatacc 240  
gcagagtcta gactcgtggt ggacttctct caattttata gggggatcac ccgtgtgtct 300  
tggccaaaat tcgcagtccc caacctcaa tcactacca acctcctgtc ctccaatttg 360  
tcctgggttat cgctggatgt gtctgcggcg ttttatcata ttctcttca tcctgtgtct 420  
atgcctcatc ttcttattgg ttctctgga ttatcaagg atgttgcccg tttgtcctct 480  
aattccagga tcaacaacaa ccagtacggg accatgcaa acctgcacga ctctgtctca 540  
aggcaactct atgtttccct catgttgctg tacaaaacct acggttgga attgcacctg 600  
tattcccatc ccctcgtcct gggctttcgc aaaataccta tgggagtggg cctcagtccg 660  
tttctcttg ctcagtttac tagtgccatt tggtcagtgg ttctgtaggc tttccccac 720  
tgtttggtt tcagctatat ggatgatgtg gtattggggg ccaagtctgt acagcatcgt 780  
gagtcccttt ataccgtgt taccaatttt cttttgtctc tgggtataca tttaaacct 840  
aacaaaacaa aaagatggg ttattcccta aacttcattg gttacataat tggaagttg 900  
ggaacattgc cacaggatca tattgtacaa aagatcaaac actgttttag aaaacttcct 960  
gttaacaggc ctattgattg gaaagtatgt caaagaattg tgggtctttt gggctttgct 1020  
gtccattta cacaatgtgg atatcctgcc ttaatgcctt tgtatgcatg tatacaggct 1080  
aaacaggctt tcactttctc gccaaacttac aaggcctttc taagtaaaca gtacatgaac 1140  
ctttaccccg ttgtcggca acggcctggg ctgtgccaa tgtttgctga cgcaaccccc 1200  
actggttggg gcttgccat aggccatcag cgcattgagtg gaacctttgt ggctcctctg 1260  
ccgatccata ctgcggaact cctagccgct tgtattgtct gcagccggtc tggagcaaag 1320  
ctcatoggaa ctgacaattc tgcgtcctc tcgcggaaat atacatcgtt tccatggctg 1380  
ctaggctgta ctgccaactg gatccttcgc gggacgtcct ttgtttacgt cccgtcggcg 1440  
ctgaatcccg cggacgacct ctctcggggc cgcttgggac tctatcgtcc cttctccgt 1500  
ctgcogttcc agccgaccac ggggcgcacc tctctttacg cggctctccc gtctgtgcct 1560  
tctcatctgc cggtcctgt gcacttcgct tcacctctgc acgttgcatg gagaccaccg 1620  
tgaacgcca tcagatcctg ccaaggtct tacataagag gactcttgga ctcccagaa 1680  
tgtcaacgac cgaccttgag gcctacttca aagactgtgt gtttaaggac tgggaggagc 1740  
tgggggagga gattagggtta aaggcttttg tattaggagg ctgtaggcat aaattggct 1800  
gcgcaccagc accatgcaac tttttcacct ctgcctaata atctcttgta catgtccac 1860

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tgttcaagcc tccaagctgt gccttgggtg gctttggggc	atg gac att gac cct	1915
	Met Asp Ile Asp Pro	
	1 5	
tat aaa gaa ttt gga gct act gtg gag tta ctc tcg ttt ttg cct tct	1963	
Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu Pro Ser		
	10 15 20	
gac ttc ttt cct tcc gtc aga gat ctc cta gac acc gcc tca gct ctg	2011	
Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser Ala Leu		
	25 30 35	
tat cga gaa gcc tta gag tct cct gag cat tgc tca cct cac cat act	2059	
Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His His Thr		
	40 45 50	
gca ctc agg caa gcc att ctc tgc tgg ggg gaa ttg atg act cta gct	2107	
Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr Leu Ala		
	55 60 65	
acc tgg gtg ggt aat aat ttg gaa gat cca gca tcc agg gat cta gta	2155	
Thr Trp Val Gly Asn Asn Leu Glu Asp Pro Ala Ser Arg Asp Leu Val		
	70 75 80 85	
gtc aat tat gtt aat act aac atg ggt tta aag atc agg caa cta ttg	2203	
Val Asn Tyr Val Asn Thr Asn Met Gly Leu Lys Ile Arg Gln Leu Leu		
	90 95 100	
tgg ttt cat ata tct tgc ctt act ttt gga aga gag act gta ctt gaa	2251	
Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg Glu Thr Val Leu Glu		
	105 110 115	
tat ttg gtc tct ttc gga gtg tgg att cgc act cct cca gcc tat aga	2299	
Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Pro Ala Tyr Arg		
	120 125 130	
cca cca aat gcc cct atc tta tca aca ctt ccg gaa act act gtt gtt	2347	
Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu Thr Thr Val Val		
	135 140 145	
aga cga cgg gac cga ggc agg tcc cct aga aga aga act ccc tcg cct	2395	
Arg Arg Arg Asp Arg Gly Arg Ser Pro Arg Arg Arg Thr Pro Ser Pro		
	150 155 160 165	
cgc aga cgc aga tct caa tcg ccg cgt cgc aga aga tct caa tct cgg	2443	
Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser Gln Ser Arg		
	170 175 180	
gaa tct caa tgt tag tattccttgg actcataagg tgggaaactt tactgggctt	2498	
Glu Ser Gln Cys		
	185	
tattcctcta cagtacctat cttaaatcct gaatggcaaa ctcttccctt tcctaagatt	2558	
catttacaag aggacattat tgataggtgt caacaatttg tgggccctct cactgtaaat	2618	
gaaaagagaa gattgaaatt aattatgcct gctagattct atcctaccca cactaaatat	2678	
ttgcccttag acaaaggaat taaaccttat tatccagatc aggtagttaa tcattacttc	2738	
caaaccagac attattttaca tactcttttg aaggctggtt ttctatataa gagggaaacc	2798	
acacgtagcg catcatcttg cgggtcacca tattcttggg aacaagagct acagcatggg	2858	

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aggttggtca ttaaaacctc gcaaaggcat ggggacgaat ctttctgttc ccaaccctct 2918  
 gggattottt cccgatcatc agttggaccc tgcattcgga gccaaactcaa acaatccaga 2978  
 ttgggacttc aaccccatca aggaccactg gccagcagcc aaccaggtag gagggggagc 3038  
 attcggggcca gggctcacc ctcacacgg cggtattttg ggggtggagcc ctcaggetca 3098  
 gggcatattg accacagtgt caacaattcc tcctcctgcc tccaccaatc ggcagtcagg 3158  
 aaggcagcct actcccatct ctccacctct aagagacagt catcctcagg ccatgcagtg 3218  
 gaa 3221

<210> 134  
 <211> 185  
 <212> PRT  
 <213> Hepatitis B virus

<400> 134  
 Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
 1 5 10 15  
 Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
 20 25 30  
 Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys  
 35 40 45  
 Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu  
 50 55 60  
 Leu Met Thr Leu Ala Thr Trp Val Gly Asn Asn Leu Glu Asp Pro Ala  
 65 70 75 80  
 Ser Arg Asp Leu Val Asn Tyr Val Asn Thr Asn Met Gly Leu Lys  
 85 90 95  
 Ile Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
 100 105 110  
 Glu Thr Val Leu Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
 115 120 125  
 Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
 130 135 140  
 Glu Thr Thr Val Val Arg Arg Arg Asp Arg Gly Arg Ser Pro Arg Arg  
 145 150 155 160  
 Arg Thr Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg  
 165 170 175  
 Arg Ser Gln Ser Arg Glu Ser Gln Cys  
 180 185

<210> 135  
 <211> 188  
 <212> PRT  
 <213> Woodchuck hepatitis B virus

<400> 135  
 Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ser Ser Tyr Gln Leu Leu  
 1 5 10 15  
 Asn Phe Leu Pro Leu Asp Phe Phe Pro Asp Leu Asn Ala Leu Val Asp  
 20 25 30  
 Thr Ala Thr Ala Leu Tyr Glu Glu Glu Leu Thr Gly Arg Glu His Cys  
 35 40 45



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Ser Pro His His Thr Ala Ile Arg Gln Ala Leu Val Cys Trp Asp Glu  
 50 55 60  
 Leu Thr Lys Leu Ile Ala Trp Met Ser Ser Asn Ile Thr Ser Glu Gln  
 65 70 75 80  
 Val Arg Thr Ile Ile Val Asn His Val Asn Asp Thr Trp Gly Leu Lys  
 85 90 95  
 Val Arg Gln Ser Leu Trp Phe His Leu Ser Cys Leu Thr Phe Gly Gln  
 100 105 110  
 His Thr Val Gln Glu Phe Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
 115 120 125  
 Pro Ala Pro Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
 130 135 140  
 Glu His Thr Val Ile Arg Arg Arg Gly Gly Ala Arg Ala Ser Arg Ser  
 145 150 155 160  
 Pro Arg Arg Arg Thr Pro Ser Pro Arg Arg Arg Ser Gln Ser Pro  
 165 170 175  
 Arg Arg Arg Arg Ser Gln Ser Pro Ser Thr Asn Cys  
 180 185

&lt;210&gt; 136

&lt;211&gt; 217

&lt;212&gt; PRT

&lt;213&gt; Ground squirrel hepatitis virus

&lt;400&gt; 136

Met Tyr Leu Phe His Leu Cys Leu Val Phe Ala Cys Val Pro Cys Pro  
 1 5 10 15  
 Thr Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Asp Met Asp  
 20 25 30  
 Ile Asp Pro Tyr Lys Glu Phe Gly Ser Ser Tyr Gln Leu Leu Asn Phe  
 35 40 45  
 Leu Pro Leu Asp Phe Phe Pro Asp Leu Asn Ala Leu Val Asp Thr Ala  
 50 55 60  
 Ala Ala Leu Tyr Glu Glu Glu Leu Thr Gly Arg Glu His Cys Ser Pro  
 65 70 75 80  
 His His Thr Ala Ile Arg Gln Ala Leu Val Cys Trp Glu Glu Leu Thr  
 85 90 95  
 Arg Leu Ile Thr Trp Met Ser Glu Asn Thr Thr Glu Glu Val Arg Arg  
 100 105 110  
 Ile Ile Val Asp His Val Asn Asn Thr Trp Gly Leu Lys Val Arg Gln  
 115 120 125  
 Thr Leu Trp Phe His Leu Ser Cys Leu Thr Phe Gly Gln His Thr Val  
 130 135 140  
 Gln Glu Phe Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Ala Pro  
 145 150 155 160

Tyr	Arg	Pro	Pro	Asn	Ala	Pro	Ile	Leu	Ser	Thr	Leu	Pro	Glu	His	Thr
				165					170					175	
Val	Ile	Arg	Arg	Arg	Gly	Gly	Ser	Arg	Ala	Ala	Arg	Ser	Pro	Arg	Arg
			180					185					190		
Arg	Thr	Pro	Ser	Pro	Arg	Arg	Arg	Arg	Ser	Gln	Ser	Pro	Arg	Arg	Arg
		195					200					205			
Arg	Ser	Gln	Ser	Pro	Ala	Ser	Asn	Cys							
	210					215									

<400>	137																
Met	Asp	Val	Asn	Ala	Ser	Arg	Ala	Leu	Ala	Asn	Val	Tyr	Asp	Leu	Pro		
1				5					10					15			
Asp	Asp	Phe	Phe	Pro	Lys	Ile	Glu	Asp	Leu	Val	Arg	Asp	Ala	Lys	Asp		
			20					25					30				
Ala	Leu	Glu	Pro	Tyr	Trp	Lys	Ser	Asp	Ser	Ile	Lys	Lys	His	Val	Leu		
		35					40					45					
Ile	Ala	Thr	His	Phe	Val	Asp	Leu	Ile	Glu	Asp	Phe	Trp	Gln	Thr	Thr		
	50					55					60						
Gln	Gly	Met	His	Glu	Ile	Ala	Glu	Ala	Ile	Arg	Ala	Val	Ile	Pro	Pro		
65					70					75					80		
Thr	Thr	Ala	Pro	Val	Pro	Ser	Gly	Tyr	Leu	Ile	Gln	His	Asp	Glu	Ala		
				85					90					95			
Glu	Glu	Ile	Pro	Leu	Gly	Asp	Leu	Phe	Lys	Glu	Gln	Glu	Glu	Arg	Ile		
			100					105					110				
Val	Ser	Phe	Gln	Pro	Asp	Tyr	Pro	Ile	Thr	Ala	Arg	Ile	His	Ala	His		
		115					120					125					
Leu	Lys	Ala	Tyr	Ala	Lys	Ile	Asn	Glu	Glu	Ser	Leu	Asp	Arg	Ala	Arg		
	130					135					140						
Arg	Leu	Leu	Trp	Trp	His	Tyr	Asn	Cys	Leu	Leu	Trp	Gly	Glu	Ala	Thr		
145					150					155					160		
Val	Thr	Asn	Tyr	Ile	Ser	Arg	Leu	Arg	Thr	Trp	Leu	Ser	Thr	Pro	Glu		
				165					170					175			
Lys	Tyr	Arg	Gly	Arg	Asp	Ala	Pro	Thr	Ile	Glu	Ala	Ile	Thr	Arg	Pro		
			180					185					190				
Ile	Gln	Val	Ala	Gln	Gly	Gly	Arg	Lys	Thr	Ser	Thr	Ala	Thr	Arg	Lys		
		195					200					205					
Pro	Arg	Gly	Leu	Glu	Pro	Arg	Arg	Arg	Lys	Val	Lys	Thr	Thr	Val	Val		
	210					215					220						
Tyr	Gly	Arg	Arg	Arg	Ser	Lys	Ser	Arg	Glu	Arg	Arg	Ala	Ser	Ser	Pro		
225					230					235					240		

Gln Arg Ala Gly Ser Pro Leu Pro Arg Ser Ser Ser Ser His His Arg  
245 250 255

Ser Pro Ser Pro Arg Lys  
260

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<210> 138
<211> 305
<212> PRT
<213> Duck hepatitis B virus
```

<400> 138  
Met Trp Asp Leu Arg Leu His Pro Ser Pro Phe Gly Ala Ala Cys Gln  
1 5 10 15

Gly Ile Phe Thr Ser Ser Leu Leu Leu Phe Leu Val Thr Val Pro Leu  
20 25 30

Val Cys Thr Ile Val Tyr Asp Ser Cys Leu Cys Met Asp Ile Asn Ala  
35 40 45

Ser Arg Ala Leu Ala Asn Val Tyr Asp Leu Pro Asp Asp Phe Phe Pro  
50 55 60

Lys Ile Asp Asp Leu Val Arg Asp Ala Lys Asp Ala Leu Glu Pro Tyr  
65 70 75 80

Trp Arg Asn Asp Ser Ile Lys Lys His Val Leu Ile Ala Thr His Phe  
85 90 95

Val Asp Leu Ile Glu Asp Phe Trp Gln Thr Thr Gln Gly Met His Glu  
100 105 110

Ile Ala Glu Ala Leu Arg Ala Ile Ile Pro Ala Thr Thr Ala Pro Val  
115 120 125

Pro Gln Gly Phe Leu Val Gln His Glu Glu Ala Glu Glu Ile Pro Leu  
130 135 140

Gly Glu Leu Phe Arg Tyr Gln Glu Glu Arg Leu Thr Asn Phe Gln Pro  
145 150 155 160

Asp Tyr Pro Val Thr Ala Arg Ile His Ala His Leu Lys Ala Tyr Ala  
165 170 175

Lys Ile Asn Glu Glu Ser Leu Asp Arg Ala Arg Arg Leu Leu Trp Trp  
180 185 190

His Tyr Asn Cys Leu Leu Trp Gly Glu Pro Asn Val Thr Asn Tyr Ile  
195 200 205

Ser Arg Leu Arg Thr Trp Leu Ser Thr Pro Glu Lys Tyr Arg Gly Lys  
210 215 220

Asp Ala Pro Thr Ile Glu Ala Ile Thr Arg Pro Ile Gln Val Ala Gln  
225 230 235 240

Gly Gly Arg Asn Lys Thr Gln Gly Val Arg Lys Ser Arg Gly Leu Glu  
245 250 255

Pro Arg Arg Arg Arg Val Lys Thr Thr Ile Val Tyr Gly Arg Arg Arg  
260 265 270

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Ser Lys Ser Arg Glu Arg Arg Ala Pro Thr Pro Gln Arg Ala Gly Ser  
           275                                  280                                  285

Pro Leu Pro Arg Thr Ser Arg Asp His His Arg Ser Pro Ser Pro Arg  
           290                                  295                                  300

Glu  
 305

<210> 139  
 <211> 212  
 <212> PRT  
 <213> Haemophilus influenzae

<400> 139  
 Met Lys Lys Thr Leu Leu Gly Ser Leu Ile Leu Leu Ala Phe Ala Gly  
   1                                  5                                  10                                  15

Asn Val Gln Ala Ala Ala Asn Ala Asp Thr Ser Gly Thr Val Thr Phe  
                                   20                                  25                                  30

Phe Gly Lys Val Val Glu Asn Thr Cys Gln Val Asn Gln Asp Ser Glu  
                                   35                                  40                                  45

Tyr Glu Cys Asn Leu Asn Asp Val Gly Lys Asn His Leu Ser Gln Gln  
           50                                  55                                  60

Gly Tyr Thr Ala Met Gln Thr Pro Phe Thr Ile Thr Leu Glu Asn Cys  
   65                                  70                                  75                                  80

Asn Val Thr Thr Thr Asn Asn Lys Pro Lys Ala Thr Lys Val Gly Val  
                                   85                                  90                                  95

Tyr Phe Tyr Ser Trp Glu Ile Ala Asp Lys Asp Asn Lys Tyr Thr Leu  
                                   100                                  105                                  110

Lys Asn Ile Lys Glu Asn Thr Gly Thr Asn Asp Ser Ala Asn Lys Val  
                                   115                                  120                                  125

Asn Ile Gln Leu Leu Glu Asp Asn Gly Thr Ala Glu Ile Lys Val Val  
           130                                  135                                  140

Gly Lys Thr Thr Thr Asp Phe Thr Ser Glu Asn His Asn Gly Ala Gly  
   145                                  150                                  155                                  160

Ala Asp Pro Val Ala Thr Asn Lys His Ile Ser Ser Leu Thr Pro Leu  
                                   165                                  170                                  175

Asn Asn Gln Asn Ser Ile Asn Leu His Tyr Ile Ala Gln Tyr Tyr Ala  
                                   180                                  185                                  190

Thr Gly Val Ala Glu Ala Gly Lys Val Pro Ser Ser Val Asn Ser Gln  
           195                                  200                                  205

Ile Ala Tyr Glu  
 210

<210> 140  
 <211> 139  
 <212> PRT  
 <213> Pseudomonas stutzeri

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&lt;400&gt; 140

Met Lys Ala Gln Met Gln Lys Gly Phe Thr Leu Ile Glu Leu Met Ile  
 1 5 10 15

Val Val Ala Ile Ile Gly Ile Leu Ala Ala Ile Ala Leu Pro Ala Tyr  
 20 25 30

Gln Asp Tyr Thr Val Arg Ser Asn Ala Ala Ala Leu Ala Glu Ile  
 35 40 45

Thr Pro Gly Lys Ile Gly Phe Glu Gln Ala Ile Asn Glu Gly Lys Thr  
 50 55 60

Pro Ser Leu Thr Ser Thr Asp Glu Gly Tyr Ile Gly Ile Thr Asp Ser  
 65 70 75 80

Thr Ser Tyr Cys Asp Val Asp Leu Asp Thr Ala Ala Asp Gly His Ile  
 85 90 95

Glu Cys Thr Ala Lys Gly Gly Asn Ala Gly Lys Phe Asp Gly Lys Thr  
 100 105 110

Ile Thr Leu Asn Arg Thr Ala Asp Gly Glu Trp Ser Cys Ala Ser Thr  
 115 120 125

Leu Asp Ala Lys Tyr Lys Pro Gly Lys Cys Ser  
 130 135

&lt;210&gt; 141

&lt;211&gt; 59

&lt;212&gt; PRT

<213> *Caulobacter crescentus*

&lt;400&gt; 141

Met Thr Lys Phe Val Thr Arg Phe Leu Lys Asp Glu Ser Gly Ala Thr  
 1 5 10 15

Ala Ile Glu Tyr Gly Leu Ile Val Ala Leu Ile Ala Val Val Ile Val  
 20 25 30

Thr Ala Val Thr Thr Leu Gly Thr Asn Leu Arg Thr Ala Phe Thr Lys  
 35 40 45

Ala Gly Ala Ala Val Ser Thr Ala Ala Gly Thr  
 50 55

&lt;210&gt; 142

&lt;211&gt; 173

&lt;212&gt; PRT

<213> *Escherichia coli*

&lt;400&gt; 142

Met Ala Val Val Ser Phe Gly Val Asn Ala Ala Pro Thr Ile Pro Gln  
 1 5 10 15

Gly Gln Gly Lys Val Thr Phe Asn Gly Thr Val Val Asp Ala Pro Cys  
 20 25 30

Ser Ile Ser Gln Lys Ser Ala Asp Gln Ser Ile Asp Phe Gly Gln Leu  
 35 40 45

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Ser Lys Ser Phe Leu Glu Ala Gly Gly Val Ser Lys Pro Met Asp Leu  
 50 55 60  
 Asp Ile Glu Leu Val Asn Cys Asp Ile Thr Ala Phe Lys Gly Gly Asn  
 65 70 75 80  
 Gly Ala Gln Lys Gly Thr Val Lys Leu Ala Phe Thr Gly Pro Ile Val  
 85 90 95  
 Asn Gly His Ser Asp Glu Leu Asp Thr Asn Gly Gly Thr Gly Thr Ala  
 100 105 110  
 Ile Val Val Gln Gly Ala Gly Lys Asn Val Val Phe Asp Gly Ser Glu  
 115 120 125  
 Gly Asp Ala Asn Thr Leu Lys Asp Gly Glu Asn Val Leu His Tyr Thr  
 130 135 140  
 Ala Val Val Lys Lys Ser Ser Ala Val Gly Ala Ala Val Thr Glu Gly  
 145 150 155 160  
 Ala Phe Ser Ala Val Ala Asn Phe Asn Leu Thr Tyr Gln  
 165 170

<210> 143  
 <211> 173  
 <212> PRT  
 <213> Escherichia coli

<400> 143  
 Met Ala Val Val Ser Phe Gly Val Asn Ala Ala Pro Thr Ile Pro Gln  
 1 5 10 15  
 Gly Gln Gly Lys Val Thr Phe Asn Gly Thr Val Val Asp Ala Pro Cys  
 20 25 30  
 Ser Ile Ser Gln Lys Ser Ala Asp Gln Ser Ile Asp Phe Gly Gln Leu  
 35 40 45  
 Ser Lys Ser Phe Leu Glu Ala Gly Gly Val Ser Lys Pro Met Asp Leu  
 50 55 60  
 Asp Ile Glu Leu Val Asn Cys Asp Ile Thr Ala Phe Lys Gly Gly Asn  
 65 70 75 80  
 Gly Ala Gln Lys Gly Thr Val Lys Leu Ala Phe Thr Gly Pro Ile Val  
 85 90 95  
 Asn Gly His Ser Asp Glu Leu Asp Thr Asn Gly Gly Thr Gly Thr Ala  
 100 105 110  
 Ile Val Val Gln Gly Ala Gly Lys Asn Val Val Phe Asp Gly Ser Glu  
 115 120 125  
 Gly Asp Ala Asn Thr Leu Lys Asp Gly Glu Asn Val Leu His Tyr Thr  
 130 135 140  
 Ala Val Val Lys Lys Ser Ser Ala Val Gly Ala Ala Val Thr Glu Gly  
 145 150 155 160  
 Ala Phe Ser Ala Val Ala Asn Phe Asn Leu Thr Tyr Gln  
 165 170

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<210> 144  
 <211> 172  
 <212> PRT  
 <213> Escherichia coli

<400> 144  
 Met Ala Val Val Ser Phe Gly Val Asn Ala Ala Pro Thr Thr Pro Gln  
     1                    5                    10                    15  
 Gly Gln Gly Arg Val Thr Phe Asn Gly Thr Val Val Asp Ala Pro Cys  
                     20                    25                    30  
 Ser Ile Ser Gln Lys Ser Ala Asp Gln Ser Ile Asp Phe Gly Gln Leu  
                     35                    40                    45  
 Ser Lys Ser Phe Leu Ala Asn Asp Gly Gln Ser Lys Pro Met Asn Leu  
     50                    55                    60  
 Asp Ile Glu Leu Val Asn Cys Asp Ile Thr Ala Phe Lys Asn Gly Asn  
     65                    70                    75                    80  
 Ala Lys Thr Gly Ser Val Lys Leu Ala Phe Thr Gly Pro Thr Val Ser  
                     85                    90                    95  
 Gly His Pro Ser Glu Leu Ala Thr Asn Gly Gly Pro Gly Thr Ala Ile  
                     100                    105                    110  
 Met Ile Gln Ala Ala Gly Lys Asn Val Pro Phe Asp Gly Thr Glu Gly  
     115                    120                    125  
 Asp Pro Asn Leu Leu Lys Asp Gly Asp Asn Val Leu His Tyr Thr Thr  
     130                    135                    140  
 Val Gly Lys Lys Ser Ser Asp Gly Asn Ala Gln Ile Thr Glu Gly Ala  
     145                    150                    155                    160  
 Phe Ser Gly Val Ala Thr Phe Asn Leu Ser Tyr Gln  
                     165                    170

<210> 145  
 <211> 853  
 <212> DNA  
 <213> Escherichia coli

<220>  
 <221> CDS  
 <222> (281)..(829)

<400> 145  
 acgtttctgt ggctcgacgc atcttctca ttcttctctc caaaaaccac ctcatgcaat 60  
 ataaacatct ataaataaag ataacaaata gaatattaag ccaacaaata aactgaaaaa 120  
 gtttggtccgc gatgctttac ctctatgagt caaatgggcc ccaatgtttc atcttttggg 180  
 ggaaactgtg cagtgttggc agtcaaactc gttgacaaac aaagtgtaca gaacgactgc 240  
 ccatgtcgat ttagaaatag ttttttgaaa ggaaagcagc atg aaa att aaa act 295  
   Met Lys Ile Lys Thr  
   1                    5  
 ctg gca atc gtt gtt ctg tcg gct ctg tcc ctc agt tct acg acg gct 343

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Leu Ala Ile Val Val Leu Ser Ala Leu Ser Leu Ser Ser Thr Thr Ala  
                                   10                                  15                                  20  
 ctg gcc gct gcc acg acg gtt aat ggt ggg acc gtt cac ttt aaa ggg 391  
 Leu Ala Ala Ala Thr Thr Val Asn Gly Gly Thr Val His Phe Lys Gly  
                                   25                                  30                                  35  
 gaa gtt gtt aac gcc gct tgc gca gtt gat gca ggc tct gtt gat caa 439  
 Glu Val Val Asn Ala Ala Cys Ala Val Asp Ala Gly Ser Val Asp Gln  
                                   40                                  45                                  50  
 acc gtt cag tta gga cag gtt cgt acc gca tcg ctg gca cag gaa gga 487  
 Thr Val Gln Leu Gly Gln Val Arg Thr Ala Ser Leu Ala Gln Glu Gly  
                                   55                                  60                                  65  
 gca acc agt tct gct gtc ggt ttt aac att cag ctg aat gat tgc gat 535  
 Ala Thr Ser Ser Ala Val Gly Phe Asn Ile Gln Leu Asn Asp Cys Asp  
                                   70                                  75                                  80                                  85  
 acc aat gtt gca tct aaa gcc gct gtt gcc ttt tta ggt acg gcg att 583  
 Thr Asn Val Ala Ser Lys Ala Ala Val Ala Phe Leu Gly Thr Ala Ile  
                                   90                                  95                                  100  
 gat gcg ggt cat acc aac gtt ctg gct ctg cag agt tca gct gcg ggt 631  
 Asp Ala Gly His Thr Asn Val Leu Ala Leu Gln Ser Ser Ala Ala Gly  
                                   105                                  110                                  115  
 agc gca aca aac gtt ggt gtg cag atc ctg gac aga acg ggt gct gcg 679  
 Ser Ala Thr Asn Val Gly Val Gln Ile Leu Asp Arg Thr Gly Ala Ala  
                                   120                                  125                                  130  
 ctg acg ctg gat ggt gcg aca ttt agt tca gaa aca acc ctg aat aac 727  
 Leu Thr Leu Asp Gly Ala Thr Phe Ser Ser Glu Thr Thr Leu Asn Asn  
                                   135                                  140                                  145  
 gga acc aat acc att ccg ttc cag gcg cgt tat ttt gca acc ggg gcc 775  
 Gly Thr Asn Thr Ile Pro Phe Gln Ala Arg Tyr Phe Ala Thr Gly Ala  
                                   150                                  155                                  160                                  165  
 gca acc ccg ggt gct gct aat gcg gat gcg acc ttc aag gtt cag tat 823  
 Ala Thr Pro Gly Ala Ala Asn Ala Asp Ala Thr Phe Lys Val Gln Tyr  
                                   170                                  175                                  180  
 caa taa cctacctagg ttcagggacg ttca 853  
 Gln

&lt;210&gt; 146

&lt;211&gt; 182

&lt;212&gt; PRT

&lt;213&gt; Escherichia coli

&lt;400&gt; 146

Met Lys Ile Lys Thr Leu Ala Ile Val Val Leu Ser Ala Leu Ser Leu  
   1                                  5                                  10                                  15  
 Ser Ser Thr Thr Ala Leu Ala Ala Ala Thr Thr Val Asn Gly Gly Thr  
                                   20                                  25                                  30  
 Val His Phe Lys Gly Glu Val Val Asn Ala Ala Cys Ala Val Asp Ala  
                                   35                                  40                                  45  
 Gly Ser Val Asp Gln Thr Val Gln Leu Gly Gln Val Arg Thr Ala Ser  
                                   50                                  55                                  60  
 Leu Ala Gln Glu Gly Ala Thr Ser Ser Ala Val Gly Phe Asn Ile Gln  
   65                                  70                                  75                                  80



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Leu Asn Asp Cys Asp Thr Asn Val Ala Ser Lys Ala Ala Val Ala Phe  
                     85                    90                    95  
 Leu Gly Thr Ala Ile Asp Ala Gly His Thr Asn Val Leu Ala Leu Gln  
                     100                    105                    110  
 Ser Ser Ala Ala Gly Ser Ala Thr Asn Val Gly Val Gln Ile Leu Asp  
                     115                    120                    125  
 Arg Thr Gly Ala Ala Leu Thr Leu Asp Gly Ala Thr Phe Ser Ser Glu  
                     130                    135                    140  
 Thr Thr Leu Asn Asn Gly Thr Asn Thr Ile Pro Phe Gln Ala Arg Tyr  
                     145                    150                    155                    160  
 Phe Ala Thr Gly Ala Ala Thr Pro Gly Ala Ala Asn Ala Asp Ala Thr  
                     165                    170                    175  
 Phe Lys Val Gln Tyr Gln  
                     180

<210> 147  
 <211> 11  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> FLAG peptide

<400> 147  
 Cys Gly Gly Asp Tyr Lys Asp Asp Asp Asp Lys  
   1                    5                    10

<210> 148  
 <211> 31  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> primer

<400> 148  
 ccggaattca tggacattga cccttataaa g 31

<210> 149  
 <211> 37  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> primer

<400> 149  
 gtgcagtatg gtgaggtgag gaatgctcag gagactc 37

<210> 150  
 <211> 37  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> primer

<400> 150

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gsgtctcctg agcattcctc acctcaccat actgcac

37

&lt;210&gt; 151

&lt;211&gt; 33

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; primer

&lt;400&gt; 151

cttccaaaag tgagggaaga aatgtgaaac cac

33

&lt;210&gt; 152

&lt;211&gt; 47

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; primer

&lt;400&gt; 152

cgcgccccaa gcttctaaac aacagtagtc tccggaagcg ttgatag

47

&lt;210&gt; 153

&lt;211&gt; 33

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; primer

&lt;400&gt; 153

gtggtttcac atttcttccc tcacttttgg aag

33

&lt;210&gt; 154

&lt;211&gt; 281

&lt;212&gt; PRT

<213> *Saccharomyces cerevisiae*

&lt;400&gt; 154

Met Ser Glu Tyr Gln Pro Ser Leu Phe Ala Leu Asn Pro Met Gly Phe  
1 5 10 15Ser Pro Leu Asp Gly Ser Lys Ser Thr Asn Glu Asn Val Ser Ala Ser  
20 25 30Thr Ser Thr Ala Lys Pro Met Val Gly Gln Leu Ile Phe Asp Lys Phe  
35 40 45Ile Lys Thr Glu Glu Asp Pro Ile Ile Lys Gln Asp Thr Pro Ser Asn  
50 55 60Leu Asp Phe Asp Phe Ala Leu Pro Gln Thr Ala Thr Ala Pro Asp Ala  
65 70 75 80Lys Thr Val Leu Pro Ile Pro Glu Leu Asp Asp Ala Val Val Glu Ser  
85 90 95

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Phe Phe Ser Ser Ser Thr Asp Ser Thr Pro Met Phe Glu Tyr Glu Asn  
                   100                  105                  110  
 Leu Glu Asp Asn Ser Lys Glu Trp Thr Ser Leu Phe Asp Asn Asp Ile  
                   115                  120                  125  
 Pro Val Thr Thr Asp Asp Val Ser Leu Ala Asp Lys Ala Ile Glu Ser  
                   130                  135                  140  
 Thr Glu Glu Val Ser Leu Val Pro Ser Asn Leu Glu Val Ser Thr Thr  
  145                  150                  155                  160  
 Ser Phe Leu Pro Thr Pro Val Leu Glu Asp Ala Lys Leu Thr Gln Thr  
                   165                  170                  175  
 Arg Lys Val Lys Lys Pro Asn Ser Val Val Lys Lys Ser His His Val  
                   180                  185                  190  
 Gly Lys Asp Asp Glu Ser Arg Leu Asp His Leu Gly Val Val Ala Tyr  
                   195                  200                  205  
 Asn Arg Lys Gln Arg Ser Ile Pro Leu Ser Pro Ile Val Pro Glu Ser  
  210                  215                  220  
 Ser Asp Pro Ala Ala Leu Lys Arg Ala Arg Asn Thr Glu Ala Ala Arg  
  225                  230                  235                  240  
 Arg Ser Arg Ala Arg Lys Leu Gln Arg Met Lys Gln Leu Glu Asp Lys  
                   245                  250                  255  
 Val Glu Glu Leu Leu Ser Lys Asn Tyr His Leu Glu Asn Glu Val Ala  
                   260                  265                  270  
 Arg Leu Lys Lys Leu Val Gly Glu Arg  
                   275                  280

&lt;210&gt; 155

&lt;211&gt; 181

&lt;212&gt; PRT

&lt;213&gt; Escherichia coli

&lt;400&gt; 155

Met Lys Ile Lys Thr Leu Ala Ile Val Val Leu Ser Ala Leu Ser Leu  
   1                  5                  10                  15  
 Ser Ser Thr Ala Ala Leu Ala Ala Ala Thr Thr Val Asn Gly Gly Thr  
                   20                  25                  30  
 Val His Phe Lys Gly Glu Val Val Asn Ala Ala Cys Ala Val Asp Ala  
                   35                  40                  45  
 Gly Ser Val Asp Gln Thr Val Gln Leu Gly Gln Val Arg Thr Ala Ser  
   50                  55                  60  
 Leu Ala Gln Glu Gly Ala Thr Ser Ser Ala Val Gly Phe Asn Ile Gln  
   65                  70                  75                  80  
 Leu Asn Asp Cys Asp Thr Asn Val Ala Ser Lys Ala Ala Val Ala Phe  
                   85                  90                  95  
 Leu Gly Thr Ala Ile Asp Ala Gly His Thr Asn Val Leu Ala Leu Gln  
                   100                  105                  110

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Ser Ser Ala Ala Gly Ser Ala Thr Asn Val Gly Val Gln Ile Leu Asp  
 115 120 125

Arg Thr Gly Ala Ala Leu Thr Leu Asp Gly Ala Thr Phe Ser Ser Glu  
 130 135 140

Thr Thr Leu Asn Asn Gly Thr Asn Thr Ile Pro Phe Gln Ala Arg Tyr  
 145 150 155 160

Phe Ala Gly Ala Ala Thr Pro Gly Ala Ala Asn Ala Asp Ala Thr Phe  
 165 170 175

Lys Val Gln Tyr Gln  
 180

&lt;210&gt; 156

&lt;211&gt; 447

&lt;212&gt; DNA

&lt;213&gt; Hepatitis B

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)..(447)

&lt;400&gt; 156

atg gac att gac cct tat aaa gaa ttt gga gct act gtg gag tta ctc	48
Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu	
1 5 10 15	
tcg ttt ttg cct tct gac ttc ttt cct tcc gta cga gat ctt cta gat	96
Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp	
20 25 30	
acc gcc gca gct ctg tat cgg gat gcc tta gag tct cct gag cat tgt	144
Thr Ala Ala Ala Leu Tyr Arg Asp Ala Leu Glu Ser Pro Glu His Cys	
35 40 45	
tca cct cac cat act gca ctc agg caa gca att ctt tgc tgg gga gac	192
Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp	
50 55 60	
tta atg act cta gct acc tgg gtg ggt act aat tta gaa gat cca gca	240
Leu Met Thr Leu Ala Thr Trp Val Gly Thr Asn Leu Glu Asp Pro Ala	
65 70 75 80	
tct agg gac cta gta gtc agt tat gtc aac act aat gtg ggc cta aag	288
Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Val Gly Leu Lys	
85 90 95	
ttc aga caa tta ttg tgg ttt cac att tct tgt ctc act ttt gga aga	336
Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg	
100 105 110	
gaa acg gtt cta gag tat ttg gtc tct ttt gga gtg tgg att cgc act	384
Glu Thr Val Leu Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr	
115 120 125	
cct cca gcc tat aga cca cca aat gcc cct atc cta tca acg ctt ccg	432
Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro	
130 135 140	
gag act act gtt gtt	447

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Glu Thr Thr Val Val  
145

&lt;210&gt; 157

&lt;211&gt; 149

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B

&lt;400&gt; 157

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
1 5 10 15

Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
20 25 30

Thr Ala Ala Ala Leu Tyr Arg Asp Ala Leu Glu Ser Pro Glu His Cys  
35 40 45

Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp  
50 55 60

Leu Met Thr Leu Ala Thr Trp Val Gly Thr Asn Leu Glu Asp Pro Ala  
65 70 75 80

Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Val Gly Leu Lys  
85 90 95

Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg  
100 105 110

Glu Thr Val Leu Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr  
115 120 125

Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro  
130 135 140

Glu Thr Thr Val Val  
145

&lt;210&gt; 158

&lt;211&gt; 152

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B

&lt;400&gt; 158

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
1 5 10 15

Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
20 25 30

Thr Ala Ala Ala Leu Tyr Arg Asp Ala Leu Glu Ser Pro Glu His Cys  
35 40 45

Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp  
50 55 60

Leu Met Thr Leu Ala Thr Trp Val Gly Thr Asn Leu Glu Asp Gly Gly  
65 70 75 80

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Lys Gly Gly Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Val  
85 90 95

Gly Leu Lys Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr  
100 105 110

Phe Gly Arg Glu Thr Val Leu Glu Tyr Leu Val Ser Phe Gly Val Trp  
115 120 125

Ile Arg Thr Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser  
130 135 140

Thr Leu Pro Glu Thr Thr Val Val  
145 150

<210> 159

<211> 56

<212> DNA

<213> Artificial Sequence

<220>

<223> Oligonucleotide

<400> 159

tagatgatta cgccaagctt ataatagaaa tagttttttg aaaggaaagc agcatg 56

<210> 160

<211> 45

<212> DNA

<213> Artificial Sequence

<220>

<223> Oligonucleotide

<400> 160

gtcaaaggcc ttgtcgacgt tattccatta cgcccgatcat tttgg 45

<210> 161

<211> 4623

<212> DNA

<213> Artificial Sequence

<220>

<223> pFIMAIC

<400> 161

agacgaaagg gcctcgtgat acgcctatatttttatagggtta atgtcatgat aataatgggtt 60

tcttagacgt cagggtggcac ttttcgggga aatgtgcgcg gaaccctat ttgtttattt 120

ttctaaatac attcaaatat gtatccgctc atgagacaat aaccctgata aatgcttcaa 180

taatattgaa aaaggaagag tatgagtatt caacatttcc gtgtcgccct tattcccttt 240

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tttgcggcat tttgccttcc tgtttttgct caccagaaa cgctggtgaa agtaaaagat	300
gctgaagatc agttgggtgc acgagtgggt tacatcgaac tggatctcaa cagcggtaag	360
atccttgaga gttttcgccc cgaagaacgt tttccaatga tgagcacttt taaagttctg	420
ctatgtggcg cggtattatc ccgtattgac gccgggcaag agcaactcgg tcgccgcata	480
cactattctc agaatgactt ggttgagtac tcaccagtca cagaaaagca tcttacggat	540
ggcatgacag taagagaatt atgcagtgct gccataacca tgagtgataa cactgcggcc	600
aacttacttc tgacaacgat cggaggaccg aaggagctaa ccgctttttt gcacaacatg	660
ggggatcatg taactcgcct tgatcgttgg gaaccggagc tgaatgaagc cataccaaac	720
gacgagcgtg acaccacgat gcctgtagca atggcaacaa cgttgcgcaa actattaact	780
ggcgaactac ttactctagc ttcccggcaa caattaatag actggatgga ggcgataaa	840
gttgaggac cacttctgcg ctcgccctt ccggctggtt ggtttattgc tgataaatct	900
ggagccggtg agcgtgggtc tcgcggtatc attgcagcac tggggccaga tggtaagccc	960
tcccgatcgt tagttatcta cagcagggg agtcaggcaa ctatggatga acgaaataga	1020
cagatcgctg agataggtgc ctactgatt aagcattggt aactgtcaga ccaagtttac	1080
tcatatatac tttagattga tttaaaactt catttttaat ttaaaaggat ctaggtgaag	1140
atcctttttg ataatctcat gacaaaaatc ccttaacgtg agttttcgtt cactgagcg	1200
tcagaccccg tagaaaagat caaaggatct tcttgagatc ctttttttct gcgcgtaatc	1260
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ctcgtctctg taatcctgtt accagtggct gctgccagtg gcgataagtc gtgtcttacc	1500
gggttgact caagacgata gttaccggat aaggcgcagc ggtcgggctg aacggggggt	1560
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gagctatgag aaagcgccac gcttcccga gggagaaagg cggacaggta tccggtaagc	1680
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tgctggcctt ttgctcacat gttctttcct gcgttatccc ctgattctgt ggataaccgt	1920
attaccgcct ttgagtgagc tgataccgct cgcgcagcc gaacgaccga gcgcagcgag	1980
tcagtgagcg aggaagcgga agagcgcca atacgaaac cgcctctccc cgcgcgttgg	2040
ccgattcatt aatgcagctg gcacgacagg tttcccgact ggaaagcggg cagtgagcgc	2100
aacgcaatta atgtgagtta gctcactcat taggcacccc aggctttaca ctttatgctt	2160

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ccggctcgta	tgttgtgtgg	aattgtgagc	ggataacaat	ttcacacagg	aaacagctat	2220
gaccatgatt	acgccaagct	tataatagaa	atagtttttt	gaaaggaaag	cagcatgaaa	2280
attaaaaactc	tggcaatcgt	tgttctgtcg	gctctgtccc	tcagttctac	agcggctctg	2340
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gcacgctgg	cacaggaagg	agcaaccagt	tctgctgtcg	gttttaacat	tcagctgaat	2520
gattgagata	ccaatgttgc	atctaaagcc	gctgttgcc	ttttaggtag	ggcgattgat	2580
gcgggtcata	ccaacgttct	ggctctgcag	agttcagctg	cgggtagcgc	aacaaacgtt	2640
ggtgtgcaga	tcctggacag	aacgggtgct	gcgctgacgc	tggatgggtc	gacatttagt	2700
tcagaaacaa	ccctgaataa	cggaaccaat	accattccgt	tcaggcgcg	ttattttgca	2760
accggggccg	caaccccg	tgctgcta	gggatgcga	ccttcaaggt	tcagtatcaa	2820
taacctacc	aggttcagg	acgtcattac	gggcagggat	gccaccctt	gtgcgataaa	2880
aataacgatg	aaaaggaaga	gattatttct	attagcgtcg	ttgctgcaa	tgtttgctct	2940
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tcatttacgg	gaatgtagca	cgggtgtgag	tgaacgtgta	ggtgtggcgt	ttcacggtgt	3180
cgcggatggt	aaaaatccg	atgtgctt	cgtgggagag	gggccaggga	tagccaccaa	3240
tattggcgta	gcgttgttg	atgatgaagg	aaacctcgta	cggattaatc	gtcctccagc	3300
aaactggaaa	cggctttatt	caggctctac	ttcgctacat	ttcatcgcca	aatatcggtc	3360
taccgggct	cgggttactg	gcggcatcgc	caatgcccag	gcctggttct	ctttaaccta	3420
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ctatttaatt	caatcatggg	tggaatatgc	cgtggtgta	aaggatggtc	gttttatcgt	3720
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cattaaactg	tactatcgcc	cggctaaatt	agcgttgcca	cccgatcagg	ccgcagaaaa	3960
attaagattt	cgtcgtagcg	cgaattctct	gacgctgatt	aaccgacac	cctattacct	4020
gacggtaaca	gagttgaatg	ccggaaccgg	ggttcttgaa	aatgcattgg	tgctccaat	4080



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gggcgaaagc acggttaaata tgccttctga tgcaggaagc aatattactt accgaacaat 4140
aaatgattat ggcgactta cccccaaaat gacgggcgta atggaataac gtcgactcta 4200
gaggatcccc gggtagcgag ctggaattca ctggccgtcg ttttacaacg tcgtgactgg 4260
gaaaaccctg gcgttaccga acttaatcgc cttgcagcac atcccccttt cgccagctgg 4320
cgtaatagcg aagaggcccg caccgatcgc ccttcccaac agttgcgcag cctgaatggc 4380
gaatggcgcc tgatgoggta ttttctcctt acgcatctgt gcggtatttc acaccgcata 4440
tggtgcactc tcagtacaat ctgctctgat gccgcatagt taagccagcc ccgacaccgc 4500
ccaacacccg ctgacgcgcc ctgacgggct tgtctgtctc cggcattccg ttacagacaa 4560
gctgtgaccg tctccgggag ctgcatgtgt cagaggtttt caccgtcatc accgaaacgc 4620
gcg 4623

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<210> 162
<211> 42
<212> DNA
<213> Artificial Sequence

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<220>
<223> Oligonucleotide

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<400> 162
aagatcttaa gctaagcttg aattctctga cgctgattaa cc 42

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<210> 163
<211> 41
<212> DNA
<213> Artificial Sequence

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<220>
<223> Oligonucleotide

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<400> 163
acgtaaagca tttctagacc gcggatagta atcgtgctat c 41

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<210> 164
<211> 5681
<212> DNA
<213> Artificial Sequence

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<220>
<223> pFIMD

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<400> 164
tcaccgtcat caccgaaacg cgcgagacga aaggccctcg tgatacgcct atttttatag 60
gttaatgtca tgataataat gggttcttag acgtcagggtg gcacttttcg gggaaatgtg 120

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cgcggaaccc ctatttgttt atttttctaa atacattcaa atatgtatcc gctcatgaga	180
caataaccct gataaatgct tcaataatat tgaaaaagga agagtatgag tattcaacat	240
ttccgtgtcg cccttattcc cttttttgcg gcattttgcc ttctgtttt tgotcaccga	300
gaaacgctgg tgaaagtaaa agatgctgaa gatcagttgg gtgcacgagt gggttacatc	360
gaactggatc tcaacagcgg taagatcctt gagagttttc gcccgaaga acgttttcca	420
atgatgagca cttttaaaagt tctgctatgt ggcgcggtat tatcccgat tgacgccggg	480
caagagcaac tcggtcgccg catacactat tctcagaatg acttggttga gtactacca	540
gtcacagaaa agcatcttac ggatggcatg acagtaagag aattatgcag tgotgccata	600
accatgagtg ataacactgc ggccaactta cttctgacaa cgatcggagg accgaaggag	660
ctaaccgctt ttttgacaa catgggggat catgtaactc gccttgatcg ttgggaaccg	720
gagctgaatg aagccatacc aaacgacgag cgtgacacca cgatgcctgt agcaatggca	780
acaacgttgc gcaaaactatt aactggcgaa ctacttactc tagcttcccg gcaacaatta	840
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ggctggttta ttgctgataa atctggagcc ggtgagcgtg ggtctcggg tatcattgca	960
gcactggggc cagatggtaa gccctcccgat atcgtagtta tctacacgac ggggagtcag	1020
gcaactatgg atgaacgaaa tagacagatc gctgagatag gtgcctcact gattaagcat	1080
tggttaactgt cagaccaagt ttactcatat atactttaga ttgatttaaa acttcatttt	1140
taatttaaaa ggatctaggt gaagatcctt tttgataatc tcatgaccaa aatcccttaa	1200
cgtgagtttt cgttccactg agcgtcagac cccgtagaaa agatcaaagg atcttcttga	1260
gatccttttt ttctgcgcgt aatctgctgc ttgcaaaca aaaaaccacc gctaccagcg	1320
gtggtttggt tgccggatca agagctacca actctttttc cgaaggtaac tggcttcagc	1380
agagcgcaga taccaaatac tgtccttcta gtgtagccgt agttaggcca ccaactcaag	1440
aactctgtag caccgcctac atacctcgtc ctgctaatac tgttaccagt ggctgctgcc	1500
agtggcgata agtcgtgtct taccgggttg gactcaagac gatagttacc ggataaggcg	1560
cagcggtcgg gctgaacggg gggttcgtgc acacagccca gcttgagcg aacgacctac	1620
accgaactga gatacctaca gcgtgagcta tgagaaagcg ccacgcttc cgaagggaga	1680
aaggcggaca ggtatccggt aagcggcagg gtcggaacag gagagcgac gagggagctt	1740
ccagggggaa acgcctggtg tctttatagt cctgtcgggt ttcgccacct ctgacttgag	1800
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tcccctgatt ctgtggataa ccgtattacc gcctttgagt gagctgatac cgctcgcgcg	1980
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ttattaccct gtttgcgtga ctgctgatgg gctggtcggt aaatgcctgg tcattcgct 8040
gtaaaaccgc caatggtacc gagctcgaat tactggccg tcgttttaca acgtcgtgac 8100
tgggaaaacc ctggcggttac ccaacttaat cgccttgag cacatcccc ttctgccagc 8160
tggcgtaata gcgaagaggc ccgcaccgat cgcccttccc aacagttgcg cagcctgaat 8220
ggcgaatggc gcctgatgcg gtattttctc cttacgcata tgtgcggtat ttcacaccgc 8280
atatggtgca ctctcagtac aatctgctct gatgccgat agttaagcca gccccgacac 8340
ccgccaacac ccgtgacgc gccctgacgg gcttgtctgc tcccggcata cgcttacaga 8400
caagctgtga ccgtctcgg gagctgcatg tgtcagaggt tttaccgct atcaccgaaa 8460
cgcg 8464

```

&lt;210&gt; 170

&lt;211&gt; 27

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Synthetic M2 Peptide

&lt;400&gt; 170

```

Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp Gly Cys
1           5           10          15

```

Arg Cys Asn Gly Ser Ser Asp Gly Gly Gly Cys

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20

25

<210> 171  
 <211> 97  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Matrix protein M2

<400> 171  
 Met Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp Gly  
 1 5 10 15  
 Cys Arg Cys Asn Gly Ser Ser Asp Pro Leu Ala Ile Ala Ala Asn Ile  
 20 25 30  
 Ile Gly Ile Leu His Leu Ile Leu Trp Ile Leu Asp Arg Leu Phe Phe  
 35 40 45  
 Lys Cys Ile Tyr Arg Arg Phe Lys Tyr Gly Leu Lys Gly Gly Pro Ser  
 50 55 60  
 Thr Glu Gly Val Pro Lys Ser Met Arg Glu Glu Tyr Arg Lys Glu Gln  
 65 70 75 80  
 Gln Ser Ala Val Asp Ala Asp Asp Gly His Phe Val Ser Ile Glu Leu  
 85 90 95  
 Glu

<210> 172  
 <211> 770  
 <212> PRT  
 <213> Homo Sapiens

<400> 172  
 Met Leu Pro Gly Leu Ala Leu Leu Leu Leu Ala Ala Trp Thr Ala Arg  
 1 5 10 15  
 Ala Leu Glu Val Pro Thr Asp Gly Asn Ala Gly Leu Leu Ala Glu Pro  
 20 25 30  
 Gln Ile Ala Met Phe Cys Gly Arg Leu Asn Met His Met Asn Val Gln  
 35 40 45  
 Asn Gly Lys Trp Asp Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile Asp  
 50 55 60  
 Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Glu Leu  
 65 70 75 80  
 Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr Ile Gln Asn  
 85 90 95

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Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe Val  
 100 105 110  
 Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu Leu  
 115 120 125  
 Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val Cys  
 130 135 140  
 Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu  
 145 150 155 160  
 Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly Ile  
 165 170 175  
 Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu  
 180 185 190  
 Ser Asp Asn Val Asp Ser Ala Asp Ala Glu Glu Asp Asp Ser Asp Val  
 195 200 205  
 Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp Lys  
 210 215 220  
 Val Val Glu Val Ala Glu Glu Glu Glu Val Ala Glu Val Glu Glu Glu  
 225 230 235 240  
 Glu Ala Asp Asp Asp Glu Asp Asp Glu Asp Gly Asp Glu Val Glu Glu  
 245 250 255  
 Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser Ile  
 260 265 270  
 Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg  
 275 280 285  
 Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala Met Ile  
 290 295 300  
 Ser Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe  
 305 310 315 320  
 Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp Thr Glu Glu Tyr  
 325 330 335  
 Cys Met Ala Val Cys Gly Ser Ala Met Ser Gln Ser Leu Leu Lys Thr  
 340 345 350  
 Thr Gln Glu Pro Leu Ala Arg Asp Pro Val Lys Leu Pro Thr Thr Ala  
 355 360 365  
 Ala Ser Thr Pro Asp Ala Val Asp Lys Tyr Leu Glu Thr Pro Gly Asp  
 370 375 380  
 Glu Asn Glu His Ala His Phe Gln Lys Ala Lys Glu Arg Leu Glu Ala  
 385 390 395 400  
 Lys His Arg Glu Arg Met Ser Gln Val Met Arg Glu Trp Glu Glu Ala  
 405 410 415  
 Glu Arg Gln Ala Lys Asn Leu Pro Lys Ala Asp Lys Lys Ala Val Ile  
 420 425 430

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Gln His Phe Gln Glu Lys Val Glu Ser Leu Glu Gln Glu Ala Ala Asn  
 435 440 445  
 Glu Arg Gln Gln Leu Val Glu Thr His Met Ala Arg Val Glu Ala Met  
 450 455 460  
 Leu Asn Asp Arg Arg Arg Leu Ala Leu Glu Asn Tyr Ile Thr Ala Leu  
 465 470 475 480  
 Gln Ala Val Pro Pro Arg Pro Arg His Val Phe Asn Met Leu Lys Lys  
 485 490 495  
 Tyr Val Arg Ala Glu Gln Lys Asp Arg Gln His Thr Leu Lys His Phe  
 500 505 510  
 Glu His Val Arg Met Val Asp Pro Lys Lys Ala Ala Gln Ile Arg Ser  
 515 520 525  
 Gln Val Met Thr His Leu Arg Val Ile Tyr Glu Arg Met Asn Gln Ser  
 530 535 540  
 Leu Ser Leu Leu Tyr Asn Val Pro Ala Val Ala Glu Glu Ile Gln Asp  
 545 550 555 560  
 Glu Val Asp Glu Leu Leu Gln Lys Glu Gln Asn Tyr Ser Asp Asp Val  
 565 570 575  
 Leu Ala Asn Met Ile Ser Glu Pro Arg Ile Ser Tyr Gly Asn Asp Ala  
 580 585 590  
 Leu Met Pro Ser Leu Thr Glu Thr Lys Thr Thr Val Glu Leu Leu Pro  
 595 600 605  
 Val Asn Gly Glu Phe Ser Leu Asp Asp Leu Gln Pro Trp His Ser Phe  
 610 615 620  
 Gly Ala Asp Ser Val Pro Ala Asn Thr Glu Asn Glu Val Glu Pro Val  
 625 630 635 640  
 Asp Ala Arg Pro Ala Ala Asp Arg Gly Leu Thr Thr Arg Pro Gly Ser  
 645 650 655  
 Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser Glu Val Lys Met Asp  
 660 665 670  
 Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys Leu  
 675 680 685  
 Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile Gly  
 690 695 700  
 Leu Met Val Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr Leu  
 705 710 715 720  
 Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly Val Val  
 725 730 735  
 Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg His Leu Ser Lys Met  
 740 745 750  
 Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys Phe Phe Glu Gln Met  
 755 760 765

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Gln Asn  
770

<210> 173

<211> 82

<212> PRT

<213> Homo Sapiens

<400> 173

Gly Ser Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser Glu Val Lys  
1 5 10 15  
Met Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln  
20 25 30  
Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile  
35 40 45  
Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val Ile Ile Ile  
50 55 60  
Thr Leu Val Met Leu Lys Lys Gln Tyr Thr Ser Asn His His Gly Val  
65 70 75 80  
Val Glu

<210> 174

<211> 42

<212> PRT

<213> Unknown

<220>

<223> Amyloid Beta Peptide

<400> 174

Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys  
1 5 10 15  
Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile  
20 25 30  
Gly Leu Met Val Gly Gly Val Val Ile Ala  
35 40

<210> 175

<211> 12

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&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; p33 peptide

&lt;400&gt; 175

Cys	Gly	Gly	Lys	Ala	Val	Tyr	Asn	Phe	Ala	Thr	Met
1				5					10		

&lt;210&gt; 176

&lt;211&gt; 37

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; DP178c peptide

&lt;400&gt; 176

Cys	Tyr	Thr	Ser	Leu	Ile	His	Ser	Leu	Ile	Glu	Glu	Ser	Gln	Asn	Gln
1				5					10					15	
Gln	Glu	Lys	Asn	Glu	Gln	Glu	Leu	Leu	Glu	Leu	Asp	Lys	Trp	Ala	Ser
			20					25					30		
Leu	Trp	Asn	Trp	Phe											
			35												

&lt;210&gt; 177

&lt;211&gt; 6

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; c-terminal linker

&lt;400&gt; 177

Gly Ser Gly Gly Cys Gly

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1 5

&lt;210&gt; 178

&lt;211&gt; 65

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; GRA2

&lt;400&gt; 178

Lys	Glu	Ala	Ala	Gly	Arg	Gly	Met	Val	Thr	Val	Gly	Lys	Lys	Leu	Ala
1				5					10					15	
Asn	Val	Glu	Ser	Asp	Arg	Ser	Thr	Thr	Thr	Thr	Gln	Ala	Pro	Asp	Ser
			20					25					30		
Pro	Asn	Gly	Leu	Ala	Glu	Thr	Glu	Val	Pro	Val	Glu	Pro	Gln	Gln	Arg
		35				40					45				
Ala	Ala	His	Val	Pro	Val	Pro	Asp	Phe	Ser	Gln	Gly	Ser	Gly	Gly	Cys
	50					55					60				

Gly  
65

&lt;210&gt; 179

&lt;211&gt; 18

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; D2 peptide

&lt;400&gt; 179

Cys	Gly	Gly	Thr	Ser	Asn	Gly	Ser	Asn	Pro	Ser	Thr	Ser	Tyr	Gly	Phe
1				5					10					15	

Ala Asn

&lt;210&gt; 180

&lt;211&gt; 18

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&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; B2 peptide

&lt;400&gt; 180

Cys Gly Gly Asp Ile Ser Asn Gly Tyr Gly Ala Ser Tyr Gly Asp Asn  
1 5 10 15

Asp Ile

&lt;210&gt; 181

&lt;211&gt; 14

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; muTNFa peptide

&lt;400&gt; 181

Cys Gly Gly Val Glu Glu Gln Leu Glu Trp Leu Ser Gln Arg  
1 5 10

&lt;210&gt; 182

&lt;211&gt; 22

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; TNFa II (3'-TNFa II)

&lt;400&gt; 182

Ser Ser Gln Asn Ser Ser Asp Lys Pro Val Ala His Val Val Ala Asn  
1 5 10 15

His Gly Val Gly Gly Cys



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20

&lt;210&gt; 183

&lt;211&gt; 20

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; TNFa II (5' TNFa II)

&lt;400&gt; 183

Cys Ser Ser Gln Asn Ser Ser Asp Lys Pro Val Ala His Val Val Ala  
 1 5 10 15  
 Asn His Gly Val  
 20

&lt;210&gt; 184

&lt;211&gt; 182

&lt;212&gt; PRT

&lt;213&gt; Escherichia coli

&lt;400&gt; 184

Met Lys Ile Lys Thr Leu Ala Ile Val Val Leu Ser Ala Leu Ser Leu  
 1 5 10 15  
 Ser Ser Thr Ala Ala Leu Ala Ala Thr Thr Val Asn Gly Gly Thr  
 20 25 30  
 Val His Phe Lys Gly Glu Val Val Asn Ala Ala Cys Ala Val Asp Ala  
 35 40 45  
 Gly Ser Val Asp Gln Thr Val Gln Leu Gly Gln Val Arg Thr Ala Ser  
 50 55 60  
 Leu Ala Gln Glu Gly Ala Thr Ser Ser Ala Val Gly Phe Asn Ile Gln  
 65 70 75 80  
 Leu Asn Asp Cys Asp Thr Asn Val Ala Ser Lys Ala Ala Val Ala Phe  
 85 90 95  
 Leu Gly Thr Ala Ile Asp Ala Gly His Thr Asn Val Leu Ala Leu Gln  
 100 105 110  
 Ser Ser Ala Ala Gly Ser Ala Thr Asn Val Gly Val Gln Ile Leu Asp

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115                      120                      125  
 Arg Thr Gly Ala Ala Leu Thr Leu Asp Gly Ala Thr Phe Ser Ser Glu  
     130                      135                      140  
 Thr Thr Leu Asn Asn Gly Thr Asn Thr Ile Pro Phe Gln Ala Arg Tyr  
     145                      150                      155                      160  
 Phe Ala Thr Gly Ala Ala Thr Pro Gly Ala Ala Asn Ala Asp Ala Thr  
                     165                      170                      175  
 Phe Lys Val Gln Tyr Gln  
                     180

&lt;210&gt; 185

&lt;211&gt; 152

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus

&lt;400&gt; 185

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu  
     1                      5                      10                      15  
 Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp  
                     20                      25                      30  
 Thr Ala Ser Ala Leu Tyr Arg Glu Ala Ile Glu Ser Pro Glu His Cys  
                     35                      40                      45  
 Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu  
                     50                      55                      60  
 Leu Met Thr Leu Ala Thr Trp Val Gly Thr Asn Leu Glu Asp Gly Gly  
     65                      70                      75                      80  
 Lys Gly Gly Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met  
                     85                      90                      95  
 Gly Leu Lys Ile Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr  
                     100                      105                      110  
 Phe Gly Arg Glu Thr Val Leu Glu Tyr Leu Val Ser Phe Gly Val Trp  
                     115                      120                      125  
 Ile Arg Thr Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser  
                     130                      135                      140  
 Thr Leu Pro Glu Thr Thr Val Val  
     145                      150

&lt;210&gt; 186

&lt;211&gt; 152

&lt;212&gt; PRT

&lt;213&gt; Hepatitis B virus



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